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I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2003903127 for a patent by GLOBAL CARDIAC SOLUTIONS PTY LTD as filed on 20 June 2003.

WITNESS my hand this
Sixteenth day of January 2004

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R/00/009
Regulation 3.2

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: **Organ preconditioning, arrest, protection, preservation and recovery**

The invention is described in the following statement:

ORGAN PRECONDITIONING, ARREST, PROTECTION, PRESERVATION AND RECOVERY

The present invention relates to a pharmaceutical composition for preconditioning, arresting, protecting and/or preserving an organ, tissue or cell. The present invention also provides a method for preconditioning, arresting, protecting and preserving organs, in particular the heart during open-heart surgery, cardiovascular diagnosis or therapeutic intervention, in particular reducing the incidences of arrhythmia, death and infarct size. The invention also relates to a method of recovering an organ from arrest, in particular after long-term arrest. The invention also provides a method for preconditioning and protecting an organ, tissue or cell from damage during therapeutic intervention and/or ischaemia, including angioplasty.

In 2000, approximately 1.2 million open-heart surgeries were performed worldwide. About 64% of these were coronary artery bypass graft procedures, 24% were heart valve replacement or repair procedures, and about 12% were related to the repair of congenital heart defects [1]. About 1.2% were neonatal. The majority of open heart surgery operations (over 80%) require cardiopulmonary bypass and elective heart arrest using a cardioplegia solution (blood or crystalloid). About 10% of patients undergoing open-heart surgery will have post-operative left-ventricular dysfunction, and up to 30% will have atrial fibrillation following surgery [2]. 3-5% of patients die in the operating room and 24% of high risk patients die within 3 years following surgery [3].

The heart may be arrested for up to 3 hours during open-heart surgery. High potassium cardioplegia (in excess of 15-20 mM) has been the basis of myocardial arrest and protection for over 40 years. Currently the majority of solutions used contain high potassium [4-6], including the widely used St Thomas No. 2 Hospital Solution which generally contains 110 mM NaCl, 16 mM KCl, 16 mM $MgCl_2$, 1.2 mM $CaCl_2$ and 10 mM $NaHCO_3$ and has a pH of about 7.8 [7]. High potassium solutions usually lead to a membrane depolarisation from about –80 to –50mV [7]. Notwithstanding hyperkalemic solutions providing acceptable clinical outcomes, recent evidence suggests that progressive potassium induced

depolarisation leads to ionic and metabolic imbalances that may be linked to myocardial stunning, ventricular arrhythmias, ischaemic injury, endothelial cell swelling, microvascular damage, cell death and loss of pump function during the reperfusion period. Infant hearts are even more prone to damage with prolonged cardioplegic arrest from high potassium than adult hearts [7-10]. The major ion imbalances postulated are linked to an increased sodium influx which in turn activates the Na^+/Ca^{2+} exchangers leading to a rise in intracellular Ca^{2+} [11]. Compensatory activation of Na^+ and Ca^{2+} ion pumps then occur, which activate anaerobic metabolism to replenish ATP with a concomitant increase in tissue lactate and fall in tissue pH [7]. Potentially damaging free radical generation and oxidative stress have also been implicated in potassium arrest and partially reversed by the administration of antioxidants. In some cases, high potassium induced ischaemia has been reported to have damaged smooth muscle and endothelial function which can compromise coronary artery flow [8].

In an attempt to minimise ischaemic damage during cardioplegic arrest, an increasing number of experimental studies have employed potassium channel openers instead of high potassium [12]. Cardioprotection using nicorandil, aprikalim or pinacidil is believed to be linked to the opening of the potassium channel which leads to a hyperpolarised state, a shortening of the action potential and decreasing Ca^{2+} influx into the cell [10]. One shortfall however is that the heart takes the same time or longer to recover with no improvement in function than with high potassium cardioplegic solutions. Another limitation is that pinacidil requires a carrier due to its low solubility in aqueous solutions. The carrier routinely used is dimethyl sulphoxide (DMSO) which is controversial when used in animal or human therapy.

Most investigators, including those who advocate using potassium channel openers, believe that as soon as blood flow is halted and the arrest solution administered, ischaemia occurs and progressively increases with time. To reduce the likelihood of damage, the applicant sought a cardioplegic solution that would place the heart in a reversible hypometabolic state analogous to the tissues of a hibernating turtle, a hummingbird in torpor or an aestivating desert frog. When

these animals drop their metabolic rate (some by over 90%), their tissues do not become progressively ischaemic but remain in a down-regulated steady state where supply and demand are matched. An ideal cardioplegic solution should produce a readily reversible, rapid electrochemical arrest with minimal tissue ischaemia. Ideally, the heart should accumulate low tissue lactate, utilise little glycogen, show minimal changes in high-energy phosphates, cytosolic redox (NAD/NADH) and the bioenergetic phosphorylation (ATP/ADP Pi) ratio and free energy of ATP. There should be little or no change in cytosolic pH or free magnesium, minimal water shifts between the intracellular and extracellular phases, and no major ultrastructural damage to organelles such as the mitochondria. The ideal cardioplegic solution should produce 100% functional recovery with no atrial fibrillation, ventricular arrhythmias, cytosolic calcium overload, or other pump abnormalities. There is no cardioplegic solution currently available which fulfils all these requirements.

In addition, ischaemic heart disease is the single leading cause of death in the US and industrialised nations [1]. Each year, about 1.1 million US people suffer a heart attack, and industry estimates there are over 2.7 million cases globally per annum. About 42% of heart attacks (ie 460,000 patients in the USA) are fatal, and half of these occur within the first hour of experiencing symptoms and before the patient reaches the hospital [1]. Ischaemia (literally "to hold back blood") is usually defined as an imbalance between blood supply and demand to an organ or tissue and results in deficient oxygen, fuel or nutrient supply to cells. The most common cause of ischaemia is a narrowing of the artery or, in the extreme case, from a blood clot blocking the artery. In 90% of cases a blood clot is usually formed from rupture of an atherosclerotic plaque.

The response of a cell to ischaemia depends upon the time and extent of the deprivation of blood supply. A large percentage of deaths from cardiac ischaemia are due to ventricular fibrillation (VF) associated with profound metabolic, ionic and functional disturbances (1). Within seconds to minutes of coronary artery occlusion there is a shift from aerobic to anaerobic metabolism, a decrease in high-energy phosphates (phosphocreatine, ATP), glycogen loss, lactate accumulation, tissue acidosis, a rise in intracellular Na^+ and Ca^{2+} and extracellular

K^+ as well as changes to the transmembrane potential and ventricular dysfunction. Restoration of coronary flow within 15 min can lead to full recovery [13,14]. However, it can also stun the myocardium leading to potentially fatal arrhythmias [15]. If ischaemia persists beyond 15 min, the deprived area of the heart will undergo a progressive loss of ATP, increased Na^+ and Ca^{2+} entry, severe membrane injury, mitochondrial dysfunction, and the closing of gap junctions between cells thereby electrically isolating the damaged cells and eventually, cell death will occur [16].

While early reperfusion remains the most effective means of salvaging the myocardium from acute ischaemia, the sudden influx of oxygen paradoxically may lead to further necrosis, ventricular arrhythmias and death [16-19]. The extent of reperfusion injury has been linked to a cascade of inflammatory reactions including the generation of cytokines, leukocytes, reactive oxygen species and free radicals [20]. Over the past decade, considerable research has focused on pharmacological strategies to protect the myocardium from ischaemia-reperfusion injury by targeting cell receptors such as adenosine, opioid, α- and β-adrenergeic, M_2 muscarinic, and endothelin-1 [21,22], ion channels (e.g. Na^+ fast, sarcolemmal K_{ATP} and mitochondrial K_{ATP}, Cl^-, and Ca^{2+}) [23], sodium-exchangers (e.g. Na^+/H^+ and Na^+/Ca^{2+}) [24] as well as heat shock proteins [25,26], nitric oxide pathways [16,22] and intracellular signalling pathways such as protein kinase C, tyrosine protein kinase, guanylate cyclase and mitogen-activated protein kinase [27].

Reperfusion of ischaemic myocardium is necessary to salvage tissue from eventual death [22,28]. However, reperfusion after even brief periods of ischaemia is associated with pathologic changes that represent either an acceleration of processes initiated during ischaemia *per se*, or new pathophysiological changes that were initiated after reperfusion. The degree and extent of "reperfusion injury" can be influenced by inflammatory responses in the myocardium. Ischaemia-reperfusion prompts a release of oxygen free radicals, cytokines and other pro-inflammatory mediators that activate both the neutrophils and the coronary vascular endothelium. The inflammatory process can lead to endothelial dysfunction, microvascular collapse and blood flow defects, myocardial infarction and apoptosis [22]. Pharmacologic anti-inflammatory therapies targeting specific

steps have been shown to decrease infarct size and myocardial injury. Adenosine and nitric oxide are two compounds which have been observed to have beneficial effects against such neutrophil-mediated inflammation.

In 1990, Homeister and colleagues aimed to limit reperfusion injury by administering an intravenous bolus of lidocaine (2 mg/kg) in open-chest dogs 1 min before a 90 min occlusion of the left circumflex coronary artery and again 1 min before reperfusion [29]. At reperfusion, adenosine was infused (150 µg/kg/ml/min) through an intracoronary catheter and continued for 1-hour reperfusion. It was concluded that the sequential treatment of lidocaine and adenosine reduced infarct size [29]. In 1996, Vander-Heide and Reimer [30] failed to reproduce these findings in the same model and concluded that intravenous adenosine therapy (150 µg/kg/ml/min) during reperfusion with or without lidocaine pretreatment did not limit infarct size after 90 min regional ischaemia. In an attempt to clarify the issue, Garratt [31] and Mahaffey, [32] administered lidocaine and adenosine *sequentially* and *separately* in humans during balloon angioplasty and thrombolytic therapy respectively, but the results were again conflicting. Garratt and colleagues [31] proposed a potential benefit in 35 patients whereas Mahaffey and colleagues, in the larger AMISTAD trials involving 236 acute myocardial infarction patients, concluded that the presence of lidocaine made no difference to the outcome of adenosine-treated patients in reducing infarct size. Indeed, the clinical outcomes of the adenosine-treated group in the AMISTAD trials tended to be slightly worse than in the placebo group [32].

The applicant previously found that the heart can be better protected by using a potassium channel opener or agonist and/or an adenosine receptor agonist (preferably adenosine) and a local anaesthetic (preferably lidocaine or lignocaine) to arrest and then preserve the heart (see WO 00/56145) [33,34], the entire disclosure of which is incorporated herein by reference.

Adenosine's actions are complex as the drug has many broad-spectrum properties. Adenosine has been shown to increase coronary blood flow [35], hyperpolarise the cell membrane, and protect during ischemia and reperfusion [22]. Adenosine also acts as a 'early' and 'delayed' preconditioning 'trigger' or agent to

protect the heart against ischemic injury [36,37]. Part of adenosine's cardioprotective properties are believed to be activation of one or more of the adenosine receptor subtypes (A1, A2a, A2b and A3) [38]. Much of adenosine's protection has been ascribed to A1 and A3 receptor activation and their associated transduction pathways leading to preconditioning, protection and preservation of cell integrity [39]. It is also known that adenosine, by activating A1 receptors, is involved in slowing the sinoatrial nodal pacemaker rate (negative chronotropy), delaying atrioventricular (A-V) nodal impulse conduction (negative dromotropy), reduces atrial contractility (negative inotropy), and inhibits the effect of catecholamines (anti-adrenergic effect) [40]. The A1-stimulated negative chronotropic, dromotropic and inotropic effects of adenosine are linked to the drug's action to reduce the activity of adenyl cyclase, to activate the inward rectifier potassium current ($I_{K\text{-}Ado}$), inhibition of phospholipid turnover, activation of ATP-sensitive K channels, inhibits effect of catecholamines on the L-type Ca^{2+} current and activation of nitric oxide synthase in AV nodal cells. A3 receptors have also shown to have direct cardioprotective effects, and A2 receptors have potent vasodilatory and anti-inflammatory actions in response to injury [22,38].

The heart possesses an extraordinary ability to 'remember' short episodes of sublethal ischaemia-reperfusion (angina) which protects the myocardium and microvascular from a subsequent lethal period of ischaemia (infarction) [41,42]. The phenomenon, known as ischaemic preconditioning, is the most powerful means of delaying cell death known. It was first described in 1986 by Murry, Jennings and Reimer who reported an infarct size reduction from 29% to 7% in anaesthetised open-chested dogs after three brief episodes of brief ischaemia followed by 40 min coronary artery occlusion [41]. Since that time, the phenomenon has been described in tissues and organs of most animal models studied [43], including human [44,45]. Two different time frames for preconditioning have been identified; an early "classical" window that lasts 1 to 3 hrs after the stimulus, and a later "delayed" window which develops over many hours and can last up to 12 to 72 hours [18,36,43,46]. The heart can also be protected from preconditioning other organs such as kidney or intestine and this phenomenon is termed remote

preconditioning [47]. Over the last two decades, the cell triggers, intracellular signaling pathways and potential end-effectors of ischaemic preconditioning have been the subject of intense investigation [48-51].

Given the reluctance of most clinicians to precondition a patient's diseased heart by temporarily tieing off the vessel in the clinical setting [45], the ultimate therapeutic goal has been to develop a pharmacological mimetic. Potential triggers include adenosine, acetylcholine, bradykinin, opiods, catecholamines, free radicals and nitric oxide [18,24,37,39, Maddock, 2002 #1313,52-54]. Another potential target are gap junctions which connect the heart cells [55]. Adenosine, however, is of particular interest because in addition to 'triggering' ischaemic preconditioning, it protects against cellular injury at multiple levels and sites from conductive tissue, myocytes through to the coronary microvasculature. Recently, adenosine's (A1) 'trigger' role in delayed pharmacological preconditioning has been linked to nitric oxide synthesis by the endothelium (eNOS), potentially representing a new pharmacological target for protecting the ischemic heart [56].

Adenosine is also known to improve myocardial recovery as an adjunct to high potassium cardioplegia [57-59]. Furthermore, adenosine can be used as a pretreatment (whether or not it is present in the arresting solution) to reduce lethal injury. In one study, adenosine was shown to rival potassium arrest solutions and more recently in blood cardioplegia, it reduced post-ischaemic dysfunction in ischaemic injured hearts [60]. Adenosine is sometimes added as an adjunct to potassium cardioplegia, but recent phase II trials were equivocal [61].

Lignocaine is a local anaesthetic which is believed to block sodium fast channels and has anti-arrhythmatic properties by reducing the magnitude of inward sodium current [62-65]. In this specification, the terms "lidocaine" and "lignocaine" are used interchangeably. The accompanying shortening of the action potential is thought to directly reduce calcium entry into the cell via Ca^{2+} selective channels and Na^{+}/Ca^{2+} exchange [65]. Recent reports also implicate lignocaine with the scavenging of free radicals such as hydroxyl and singlet oxygen in the heart during reperfusion [66]. Associated with this scavenging

function, lignocaine may also inhibit phospholipase activity and minimise membrane degradation during ischaemia. Lignocaine can also depress vascular relaxations by a complex mechanism including poly(ADP-ribose) synthetase enzyme activity, but this effect has recently been shown to be pH dependent with little inhibition occurring below pH 7.2 . Lignocaine's vasodilatory effects are believed due to calcium entry blockade that do not appear to involve Na^+ channel blockade or opening of K^+-channels [67]. Lignocaine has also been shown to have a myocardial protective effect and in one study was found to be superior to high potassium solutions. However, these experiments show that lignocaine alone at 0.5, 1.0 and 1.5 mM gave highly variable functional recoveries using the isolated working rat heart. Lignocaine has also been shown to reduce infarct size in the brain and protect against reperfusion injury in the heart. More recently lignocaine has been shown to exhibit a number of pharmacological actions not related to the sodium channel block. For example, recent work has shown that local anaesthetics, including lignocaine, inhibit inflammatory responses [68,69]. They also have beneficial effects in a number of pathological processes dependent on an overly active inflammatory response such as adult respiratory distress syndrome and in ischaemia-reperfusion injury. Intravenous lignocaine has also been shown to be effective in prevention of deep vein thrombosis after elective hip surgery [70]. Lignocaine therefore appears to be effective in both attenuating inflammatory and hypercoagulable states (post-operative thrombosis) in the clinical setting [70,71]. Unlike adenosine, lignocaine has not been implicated in the preconditioning of a cell, tissue or organ.

However, the combination of the potassium channel opener and local anaesthetic result in arrest and better protection of the organ under normal potassium concentration (ie, physiological levels of potassium), thus reducing the risk of potassium induced injury to the organ which prior art high potassium arrest solutions may induce. This cardioplegia solution containing the combination of the potassium channel opener and local anaesthetic was shown by the applicant to generally improve functional recovery from arrest of the organ over existing solutions.

This invention is directed towards overcoming, or at least alleviating, one or more of the difficulties or deficiencies associated with the prior art.

In particular, this invention is intended to provide a pharmaceutical composition for preconditioning, arresting, protecting and/or preserving an organ, tissue or cell with improved recovery of, and/or reduced damage to, an organ, after arrest of the organ. It is also directed to providing a method for preconditioning, arresting, protecting and/or preserving an organ, tissue or cell.

Accordingly, in one aspect of the present invention there is provided a method for preconditioning, protecting and/or reducing damage to an organ, tissue or cell during ischaemia comprising delivering an effective amount of:

a potassium channel opener and/or adenosine receptor agonist; and

a local anaesthetic.

The applicant has surprisingly found that the simultaneous delivery of a solution including these two components prior to, during or following ischaemia markedly reduces cell damage during ischaemia. In particular, continuous administration of a solution (which may be carried in physiological saline or compatible fluid (eg, patient's own blood)) of the two components results in significantly less damage to heart tissue than delivery of the components of the composition separately (eg. one component (adenosine) parenterally and the other (lignocaine) in intermittent bolus doses).

The simultaneous delivery of the two components briefly prior to ischaemia, throughout ischaemia and reperfusion shows surprisingly increased efficacy.

In another aspect of the invention, there is provided a method of reducing myocardial tissue damage during a heart attack or cardioplegia by delivering a composition to the tissue, the composition comprising an effective amount of a potassium channel opener and/or adenosine receptor agonist; a local anaesthetic; and physiological saline or blood.

In another aspect of the invention, there is provided a method of protecting myocardial tissue from reperfusion injury, including inflammatory and blood coagulation effects often experienced during reperfusion following an ischaemic event. The method comprises administering a solution comprising an effective amount of a potassium channel opener and/or adenosine receptor agonist and a local anaesthetic.

The invention also provides a method for reducing infarction size and/or reducing inflammation and blood coagulation responses in myocardial tissue during ischaemia and/or reperfusion comprising administration of the same solution.

The invention also provides a method for reducing electrical disturbances in the heart such as atrial or ventricular arrhythmias (including lethal ventricular tachycardias and ventricular fibrillation) during ischaemia and/or reperfusion comprising administration of the same solution.

The pharmaceutical composition of the present invention protects the organ after arrest of the organ, with good to excellent recoveries of function after reperfusion.

The invention also provides a use of the composition as described above (together with the preferred embodiments described below) in the methods described above. This use of the composition can extend to many therapeutic applications, including without limitation, cardiovascular diagnosis (including coronary angiography, myocardial scintigraphy, non-invasive diagnosis of dual AV nodal conduction), use in treatment of heart attack, resuscitation therapy, short-term and long-term storage of organs tissues or cells (including graft vessels), use before, prior to, during or following open-heart surgery, angioplasty and other therapeutic interventions.

In one embodiment, the composition comprises adenosine and lignocaine. In particular, the composition may include adenosine and lignocaine in the weight ratio of about 1:2.

The composition can be infused or administered as a bolus intravenous, intracoronary or any other suitable delivery route as pretreatment for protection during a cardiac intervention such as open heart surgery (on-pump and off-pump), angioplasty (balloon and with stents or other vessel devices) and as with clot-busters (anti-clotting drug or agents).

The composition can also be infused or administered as a bolus intravenous, intracoronary or any other suitable delivery route for protection during a cardiac intervention such as open heart surgery (on-pump and off-pump), angioplasty (balloon and with stents or other vessel devices) and as with clot-busters to protect and preserve the cells from injury.

The composition may also be infused or administered as a bolus intravenous, intracoronary or any other suitable delivery route for protection following a cardiac intervention such as open heart surgery (on-pump and off-pump), angioplasty (balloon and with stents or other vessel devices) and as with clot-busters to protect and preserve the cells from injury.

In this application, without being bound by this mode of action, protection is thought to involve a multi-tiered system from modulating membrane excitability to a multitude of intracellular signaling pathways leading to (i) reduced ion imbalances, in particular sodium and calcium ion loading in the cells, (ii) improved atrial and ventricular matching of electrical conduction to metabolic demand, which may involve modulation of gap junction communication, (iii) vasodilation of coronary arteries and (ii) attenuation of the inflammatory response to injury

Infusion of the composition during pretreatment and ischaemia and reperfusion provides continuous protection from ischaemic tissue injury including protection from lethal arrhythmias. The protection from localised injury and inflammation can also be obtained when placing a stent into a vessel such as during angioplasty. The composition is also used within a polymer or special coating for a stent for use in any vessel of the body including coronary arteries, carotid arteries, or leg arteries of the body.

The composition according to the invention includes a potassium channel opener. Potassium channel openers are agents which positively act on the channel to open it. This results in efflux of potassium ions across the membrane out of the cell of the tissue. It will be appreciated that the potassium channel openers include the potassium channel agonists which also stimulate the activity of the potassium channel with the same result. It will also be appreciated that the potassium channel openers include compounds which act both directly and indirectly on the potassium channel opener resulting in the opening of the channel.

The potassium channel opener may be selected from the group consisting of: nicorandil, diazoxide, minoxidil, pinicadil, aprikalim, cromokulim, acetlycholine, NS-1619 (1,3-dihydro-1-[2-hydroxy5(trifluoromethyl)phenyl]5-(trifluoromethyl)2-H-benimidazol-one), amlodipine, Bay K 8644(L-type)(1,4-dihydro-26-dimethyl-5-nitro-4[2(trifluoromethyl)phenyl]-3-pyridine carboxylic acid (methyl ester)), bepridil HCl (L-type), calciseptine (L-type), omega-conotoxin GVIA (N-type), omega-conotoxin MVIIC (Q-type), cyproheptadine HCl, dantrolene sodium (Ca^{2+} release inhibitor), diltiazem HCl (L-type), filodipine, flunarizine HCl (Ca^{2+}/Na^{+}), fluspirilene (L-type), HA-1077 2HCl(1-(5 isoquinolinyl sulphonyl) homo piperazine.HCl), isradipine, loperamide HCl, manoalide (Ca^{2+} release inhibitor), nicardipine HCl (L-type), nifedipine (L-type), niguldipine HCl (L-type), nimodipine (L-type), nitrendipine (L-type), pimozide (L- and T-type), ruthenium red, ryanodine (SR channels), taicatoxin, verapamil HCl (L-type), methoxy-verapamil HCl (L-type), amlodipine, felodipine, YS-035 HCl (L-type)N[2(3,4-dimethoxyphenyl)ethyl]-3,4-dimethoxy N-nethyl benzene ethaneamine HCl) and AV blockers such as verapamil and adenosine. It will be appreciated that this list includes calcium antagonists as potassium channel openers are indirect calcium antagonists.

Adenosine is particularly preferred as the potassium channel opener or agonist. Adenosine is capable of opening the potassium channel, hyperpolarising the cell, depressing metabolic function, possibly protecting endothelial cells, enhancing preconditioning of tissue and protecting from ischaemia or damage. Adenosine is also an indirect calcium antagonist, vasodilator, antiarrhythmic, antiadrenergic, free radical scavenger, arresting agent, anti-inflammatory agent

(attenuates neutrophil activation), analgesic, metabolic agent and possible nitric oxide donor.

It will be appreciated that anti-adrenergics such as beta-blockers, for example, esmolol, atenolol, metoprolol and propranolol could be used instead of or in combination with the potassium channel opener to reduce calcium entry into the cell. Preferably, the beta-blocker is esmolol. Similarly, alpha(1)-adrenoceptor-antagonists such as prazosin, could be used instead of or in combination with the potassium channel opener to reduce calcium entry into the cell and therefore calcium loading.

Thus, in another aspect of the invention there is provided a method for preconditioning, protecting and/or reducing damage to an organ, tissue or cell during ischemia comprising delivery of an effective amount of:

an antiadrenergic; and

a local anaesthetic.

According to this aspect of the present invention there is also provided a composition including an effective amount of an antiadrenergic and a local anaesthetic.

Adenosine is also known to indirectly inhibit the sodium-calcium exchanger which would reduce cell sodium and calcium loading. It will be appreciated that inhibitors of the sodium-calcium exchanger would lead to reduced calcium entry and magnify the effect of adenosine. Na^+/Ca^{2+} exchange inhibitors may include benzamyl, KB-R7943 (2-[4-(4-Nitrobenzyloxy)phenyl]ethyl]isothiourea mesylate) or SEA0400 (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline).

Since one of adenosine's properties is to reduce calcium entry and sodium entry in the heart and coronary vascular cells, it will be further appreciated that a compound leading to reduced calcium and sodium entry (or reduce calcium oscillations in the cell) before, during and/or following treatment could be used instead of or in combination with adenosine to reduce calcium entry into the cell. Such compounds may be selected from calcium blockers from three different classes: 1,4-dihydropyridines (eg. nitrendipine), phenylalkylamines (eg. verapamil), and the benzothiazepines (e.g. diltiazem, nifedipine). Calcium

blockers can be selected from nifedipine, nicardipine, nimodipine, nisoldipine, lercanidipine, telodipine, angizem, altiazem, bepridil, amlodipine, felodipine, isradipine and cavero and other racemic variations. In addition, it will be appreciated that calcium entry could be inhibited by other calcium blockers which could be used instead of or in combination with adenosine and include a number of venoms from marine or terrestrial animals such as the omega-conotoxin GVIA (from the snail *conus geographus*) which selectively blocks the N-type calcium channel or omega-agatoxin IIIA and IVA from the funnel web spider *Agelelnopsis aperta* which selectively blocks R- and P/Q-type calcium channels respectively. There are also mixed voltage-gated calcium and sodium channel blockers such as NS-7 which could also be used instead of or in combination with adenosine to reduce calcium and sodium entry and thereby assist cardioprotection.

In another embodiment, the pharmaceutical composition according to the invention further includes an additional potassium channel opener. Preferably the additional potassium channel opener is diazoxide. Diazoxide is believed to preserve ion and volume regulation, oxidative phosphorylation and mitochondrial membrane integrity (appears concentration dependent). Diazoxide also affords cardioprotection by reducing mitochondrial oxidant stress at reoxygenation [81]. There is also some evidence that the protective effects of potassium channel openers are associated with modulation of reactive oxygen species generation in mitochondria [42,49].

The composition according to the invention includes an adenosine receptor agonist. It will be appreciated that the adenosine receptor agonists include compounds which act both directly and indirectly on the receptor resulting in activation of the receptor, or mimic the action of the receptor having the same net effect.

Suitable adenosine receptor agonists can be found in the reviews by Linden and colleagues [38,72], Hayes [72] and Belardinelli [73]. They may be selected from: N^6-cyclopentyladenosine (CPA), N-ethylcarboxamido adenosine (NECA), 2-[p-(2-carboxyethyl)phenethyl-amino-5'-N-ethylcarboxamido adenosine (CGS-21680), 2-chloroadenosine, N^6-[2-(3,5-demethoxyphenyl)-2-(2-

methoxyphenyl]ethyladenosine, 2-chloro-N^6-cyclopentyladenosine (CCPA), N-(4-aminobenzyl)-9-[5-(methylcarbonyl)-beta-D-robofuranosyl]-adenine (AB-MECA), ([IS-[1a,2b,3b,4a(S*)]]-4-[7-[[2-(3-chloro-2-thienyl)-1-methyl-propyl]amino]-3H-imidazole[4,5-b]pyridyl-3-yl]cyclopentane carboxamide (AMP579), N^6-(R)-phenylisopropyladenosine (R-PLA), aminophenylethyladenosine 9APNEA) and cyclohexyladenosine (CHA) [72]. Others include full adenosine A1 receptor agonists such as N-[3-(R)-tetrahydrofuranyl]-6-aminopurine riboside (CVT-510), or partial agonists such as CVT-2759 and allosteric enhancers such as PD81723 [74-76]. Other agonists include N6-cyclopentyl-2-(3-phenylaminocarbonyltriazene-1-yl)adenosine (TCPA), a very selective agonist with high affinity for the human adenosine A1 receptor [77], and allosteric enhancers of A1 adenosine receptor includes the 2-amino-3-naphthoylthiophenes [78].

CCPA is a particularly preferred adenosine receptor agonist. CCPA an A1 adenosine receptor agonist.

Modulation of agonist responses at the A1 adenosine receptor can also be achieved indirectly by an irreversible antagonist, receptor-G protein uncoupling and by the G protein activation state [79]. Thus any agonist or antagonist which modulates the G protein activation state may be used to mimic adenosine receptor activation. There is also some evidence that there is some cross-talk between adenosine receptors. Furthermore, there is data suggesting that there are converging pathways and/or receptor cross-talk between adenosine 1 (and perhaps A3) receptors and delta1-opioid receptor mediated cardioprotection [80]. Thus opiod receptor activation may result in identical protection as A1 receptor activation and therefore opiod receptors plus lidocaine would be expected to have similar effects as potassium channel opener or adenosine or adenosine agonists plus lidocaine.

Local anaesthetic agents are drugs which are used to produce reversible loss of sensation in an area of the body. Many local anaesthetic agents consist of an aromatic ring linked by a carbonyl containing moiety through a carbon chain to a substituted amino group. In general there are 2 classes of local anaesthetics

defined by their carbonyl-containing linkage group. The ester agents include cocaine, amethocaine, procaine and chloroprocaine, whereas the amides include prilocaine, mepivacaine, bupivacaine, mexiletine and lignocaine. At high concentrations, many drugs that are used for other purposes possess local anaesthetic properties. These include opioid analgesics, Beta-adrenoceptor antagonists, anticonvulsants (lamotrigine and lifarizine) and antihistamines. The local anaesthetic component of the pharmaceutical composition according to the present invention may be selected from these classes, or derivatives thereof, or from drugs than may be used for other purposes. Preferably, the component possesses local anaesthetic properties also.

Preferably the local anaesthetic is Lignocaine. Lignocaine is preferred as it is capable of acting as a local anaesthetic probably by blocking sodium fast channels, depressing metabolic function, lowering free cytosolic calcium, protecting against enzyme release from cells, possibly protecting endothelial cells and protecting against myofilament damage. At lower therapeutic concentrations lidocaine normally has little effect on atrial tissue, and therefore is ineffective in treating atrial fibrillation, atrial flutter, and supraventricular tachycardias [65]. Lignocaine is also a free radical scavenger, an antiarrhythmic and has anti-inflammatory and anti-hypercoagulable properties. It must also be appreciated that at non-anaesthetic therapeutic concentrations, local anaesthetics like lidocaine would not completely block the voltage-dependent sodium fast channels, but would down-regulate channel activity and reduce sodium entry [82,83]. As anti-arrhythmic, lidocaine is believed to target small sodium currents that normally continue through phase 2 of the action potential and consequently shortens the action potential and the refractory period [65].

As lignocaine acts as a local anaesthetic by primarily blocking sodium fast channels, it will be appreciated that other sodium channel blockers could be used instead of or in combination with the local anaesthetic in the method and composition of the present invention. Examples of suitable sodium channel blockers include venoms such as tetrodotoxin, and the drugs primaquine, QX, HNS-32 (CAS Registry # 186086-10-2), NS-7, kappa-opioid receptor agonist U50 488, crobenetine, pilsicainide, phenytoin, tocainide, mexiletine, RS100642,

riluzole, carbamazepine, flecainide, propafenone, amiodarone, sotalol, imipramine and moricizine, or any of derivatives thereof.

In another embodiment of the present invention there is provided a pharmaceutical composition according to the present invention, further including an effective amount of an antioxidant.

The antioxidant component of the pharmaceutical composition according to the present invention may be selected from one or more of the group consisting of: allopurinol, carnosine, Coenzyme Q 10, n-acetyl-cysteine, superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GP), catalase and the other metalloenzymes, glutathione, U-74006F, vitamin E, Trolox (soluble form of vitamin E), Vitamin C, Beta-Carotene (plant form of vitamin A), selenium, Gamma Linoleic Acid (GLA), alpha-lipoic acid, uric acid (urate), curcumin, bilirubin, proanthocyanidins, epigallocatechin gallate, Lutein, lycopene, bioflavonoids and polyphenols.

Preferably, the antioxidant is allopurinol. Allopurinol is a competitive inhibitor of the reactive oxygen species generating enzyme xanthine oxidase. Allopurinol's antioxidative properties may help preserve myocardial and endothelial functions by reducing oxidative stress, mitochondrial damage, apoptosis and cell death.

In another embodiment of the present invention there is provided a pharmaceutical composition according to the present invention, further including an effective amount of a sodium hydrogen exchange inhibitor. The sodium hydrogen exchange inhibitor reduces sodium and calcium entering the cell.

The sodium hydrogen exchange inhibitor may be selected from one or more of the group consisting of amiloride, cariporide, eniporide, triamterene and EMD 84021, EMD 94309, EMD 96785 and HOE 642 and T-162559 (inhibitors of the isoform 1 of the Na^+/H^+ exchanger). Preferably, the sodium hydrogen exchange inhibitor is amiloride. Amiloride inhibits the sodium proton exchanger (Na^+/H^+ exchanger, also often abbreviated NHE-1) and reduces calcium entering the cell.

During ischaemia excess cell protons (or hydrogen ions) are exchanged for sodium via the Na^+/H^+ exchanger.

In yet another embodiment of the present invention there is provided a pharmaceutical composition according to the present invention, further including an effective amount of:

a source of magnesium in an amount for increasing the amount of magnesium in a cell in the tissue; and

a source of calcium in an amount for increasing the amount of calcium in a cell in the tissue.

Elevated magnesium and low calcium has been associated with protection during ischaemia and reoxygenation of the organ. The action is believed due to decreased calcium loading.

Preferably the magnesium is present at a concentration of between 0.5mM to 20mM, more preferably about 2.5mM. Preferably the calcium present is at a concentration of between 0.1mM to 2.5mM, more preferably about 0.3mM.

In another preferred embodiment of the present invention, there is provided a pharmaceutical composition according to the present invention including an effective amount of:

a potassium channel opener and/or adenosine receptor agonist; and

a local anaesthetic,

and further including an effective amount of one or more components selected from:

diazoxide;

an antioxidant;

a sodium hydrogen exchange inhibitor;

a source of magnesium; and

a source of calcium.

The term "organ" is used herein in its broadest sense and refers to any part of the body exercising a specific function including tissues and cells or parts thereof, for example, cell lines or organelle preparations. Other examples include conduit vessels such as arteries or veins or circulatory organs such as the heart, respiratory organs such as the lungs, urinary organs such as the kidneys or bladder, digestive organs such as the stomach, liver, pancreas or spleen, reproductive organs such as the scrotum, testis, ovaries or uterus, neurological organs such as the brain, germ cells such as spermatozoa or ovum and somatic cells such as skin cells, heart cells (ie, myocytes), nerve cells, brain cells or kidney cells.

It will be understood that the term "comprises" or its grammatical variants as used in this specification and claims is equivalent to the term "includes" and is not to be taken as excluding the presence of other elements or features.

The pharmaceutical composition of the present invention is particularly useful in preconditioning, arresting, protecting and/or preserving the heart during open-heart surgery including heart transplants. Other applications include reducing heart damage before, during or following cardiovascular intervention which may include a heart attack, "beating heart" surgery, angioplasty or angiography. For example, the composition could be administered to subjects who have suffered or are developing a heart attack and used at the time of administration of blood clot-busting drugs such as streptokinase. As the clot is dissolved, the presence of the composition may protect the heart from further injury such as reperfusion injury. The pharmaceutical composition may be particularly effective as a cardioprotectant in those portions of the heart that have been starved of normal flow, nutrients and/or oxygen for different periods of time. For example, the pharmaceutical composition may be used to treat heart ischaemia which could be pre-existing or induced by cardiovascular intervention.

In a preferred embodiment the composition according to the present invention is a cardioplegic and/or cardioprotectant composition.

According to another aspect of the present invention there is provided use of the pharmaceutical composition according to the present invention in the manufacture of a medicament for preconditioning, arresting, protecting and/or preserving an organ.

In a preferred embodiment of this aspect of the present invention it is preferred to aerate the pharmaceutical composition with a source of oxygen before and/or during use. The source of oxygen may be an oxygen gas mixture where oxygen is the predominant component. The oxygen may be mixed with, for example CO_2. Preferably, the oxygen gas mixture is 95% O_2 and 5% CO_2.

It is considered that the oxygenation with the oxygen gas mixture maintains mitochondrial oxidation and this helps preserve the myocyte and endothelium of the organ.

In another aspect of the present invention there is provided a method for preconditioning, arresting, protecting and/or preserving an organ including:

providing a pharmaceutical composition including an effective amount of

a potassium channel opener and/or adenosine receptor agonist, and a local anaesthetic in a suitable container; and

a source of oxygen;

aerating the pharmaceutical composition with the oxygen; and

placing the organ in contact with the pharmaceutical composition under conditions sufficient to arrest, protect and/or preserve thereof.

Preferably the oxygen source is an oxygen gas mixture. Preferably oxygen is the predominant component. The oxygen may be mixed with, for example CO_2. More preferably, the oxygen gas mixture is 95% O_2 and 5% CO_2.

Preferably the pharmaceutical composition is aerated before and/or during contact with the organ.

Preferably the pharmaceutical composition according to this aspect of the invention is in liquid form. Liquid preparations of the pharmaceutical composition may take the form of, for example, solutions, syrups, or suspensions, or may be presented as a dry product for constitution with water or other suitable vehicle. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents, emulsifying agents, non-aqueous vehicles, preservatives and energy sources.

According to this aspect of the present invention there is provided a method for preconditioning, arresting, protecting and/or preserving an organ, wherein the pharmaceutical composition further includes an effective amount of one or more components selected from:

diazoxide;

an antioxidant;

a sodium hydrogen exchange inhibitor;

a magnesium source; and

a calcium source.

While the present invention is particularly advantageous in preconditioning, arresting, protecting and/or preserving an organ while intact in the body of a subject, for example in the treatment of the heart in circumstances of myocardial infarction or heart attack, it will also be appreciated that the present invention may also be used to arrest, protect and/or preserve isolated organs.

The subject may be a human or an animal such as a livestock animal (eg. sheep, cow or horse), laboratory test animal (eg. mouse, rabbit or guinea pig) or a companion animal (eg. dog or cat), particularly an animal of economic importance.

The method of the present invention involves contacting an organ with the pharmaceutical composition, for a time and under conditions sufficient for the organ to be arrested, protected and/or preserved.

While it is possible for each component of the pharmaceutical composition to contact the organ alone, it is preferable that the components of the pharmaceutical composition be provided together with one or more pharmaceutically acceptable carriers, diluents, adjuvants and/or excipients. Each carrier, diluent, adjuvant and/or excipient must pharmaceutically acceptable such that they are compatible with the components of the pharmaceutical composition and not harmful to the subject. Preferably, the pharmaceutical composition is prepared with liquid carriers, diluents, adjuvants and/or excipients.

Accordingly, this aspect of the invention also provides a method for preconditioning, arresting, protecting and/or preserving an organ, which includes providing the pharmaceutical composition together with a pharmaceutically acceptable carrier, diluent, adjuvant and/or excipient.

A preferred pharmaceutically acceptable carrier is a buffer having a pH of about 6 to about 9, preferably about 7, more preferably about 7.4 and/or low concentrations of potassium, for example, up to about 10mM, more preferably about 2 to about 8 mM, most preferably about 4 to about 6mM. Suitable buffers include Krebs-Henseleit which generally contains 10mM glucose, 117 mM NaCl, 5.9 mM KCl, 25 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1.12 mM$CaCl_2$ (free Ca^{2+}=1.07mM) and 0.512 mM $MgCl_2$ (free Mg^{2+}=0.5mM), St. Thomas No. 2 solution, Tyrodes solution which generally contains 10mM glucose, 126 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.33 mM NaH_2PO_4 and 10 mM HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethane sulphonic acid]. Fremes solution, Hartmanns solution which generally contains 129 NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 29 mM lactate and Ringers-Lactate. One advantage of using low potassium

is that it renders the present composition less injurious to the subject, in particular paediatric subjects such as neonates/infants. High potassium has been linked to an accumulation of calcium which may be associated with irregular heart beats during recovery, heart damage and cell swelling. Neonates/infants are even more susceptible than adults to high potassium damage during cardiac arrest. After surgery for defects a neonate/infant's heart may not return to normal for many days, sometimes requiring intensive therapy or life support. It is also advantageous to use carriers having low concentrations of magnesium, such as, for example up to about 2.5mM, but it will be appreciated that high concentrations of magnesium, for example up to about 20mM, can be used if desired without substantially affecting the activity of the composition.

In another embodiment of the present invention there is provided use of a pharmaceutical composition for preconditioning, arresting, protecting and/or preserving an organ including an effective amount of:

a potassium channel opener and/or adenosine receptor agonist and;

a local anaesthetic;

provided in a suitable container together with a source of oxygen;

wherein the pharmaceutical composition is aerated with the oxygen and contacts the organ.

Preferably the oxygen source is an oxygen gas mixture. Preferably, oxygen is the predominant component. The oxygen may be mixed with for example CO_2. More preferably, the oxygen gas mixture is 95% O_2 and 5% CO_2.

Preferably the pharmaceutical composition is aerated before and/or during contact with the organ.

In another aspect of this embodiment of the invention, there is provided a system for preconditioning, arresting, protecting and/or preserving an organ, including a pharmaceutical composition including an effective amount of:

a potassium channel opener and/or adenosine receptor agonist; and

a local anaesthetic;

the pharmaceutical composition further including effective amounts of one or more components selected from:

diazoxide;

an antioxidant;

a sodium hydrogen exchange inhibitor;

a magnesium source; and

a calcium source,

in combination with a source of oxygen.

In another preferred embodiment of the present invention there is also provided a reperfusion solution which is administered after long-term arrest protection and preservation with the solution according to the invention.

Preferably, the reperfusion solution is Krebs Henseleit buffer. More preferably, the Krebs Henseleit buffer includes an effective amount of one or more components selected from :

a magnesium source and a calcium source;

an antioxidant;

a sodium hydrogen exchange inhibitor; and

an energy substrate.

Preferably, the reperfusion solution is provided at 37°C.

The energy substrate helps with recovering metabolism. The energy substrate can be selected from one or more components selected from the group consisting of: pyruvate, glutamate, aspartate, arginine, lactate, glucose, insulin, alpha-keto glutarate, malate, succinate.

The invention will now be described with reference to the following examples. These examples are not to be construed as limiting in any way. In this description, there are the following figures.

Figure 1. Illustrates diagrammatically the experimental design described in more detail below.

Figure 2. Arrhythmic deaths from ventricular fibrillation during LCA occlusion.

Figure 3. The episodes and duration of ventricular tachycardia (VT) and ventricular fibrillation (VF) and VT+VF during ischaemia for surviving rats in all treatment groups. These values represent overall sum of episodes and durations (sec) that occurred throughout the 30 min ischaemic period. The percentage of animals that experienced either VT or VF per group are shown. Surviving rats: saline-control, n=5, AL solution, n=7; Ado-only, n=4; Lido-only, n=6. *P<0.05 vs. control, †P<0.05 vs AL-I group.

Figure 4. Effects of AL mixture, adenosine alone or lidocaine alone treatments on left ventricle necrosis and infarct size. Areas at risk (AAR/LV) were not significantly different between groups (A). Areas of necrosis in the left ventricle (AN/LV) were significantly smaller with AL mixture treatment (B). Infarct sizes (AN/AAR) in groups receiving AL solution treatment were significantly smaller compared with all other treatment groups (C). Only data from Surviving rats are shown: saline-control, n=5; AL soln, n=7; Ado-only, n=4; Lido-only, n=6. *P<0.05 vs. control, ŸP<0.05 vs AL-soln group. The black filled-in squares represent the mean ± SEM for each of the groups whereas the open symbols represent the values for each animal in that group.

Figure 5. Hemodynamic changes for all surviving animals during the course of the first experiment. Measurements were recorded throughout

pretreatment/preocclusion, ischaemia and reperfusion. Shown above are in order of appearance: equilibration, following 5 min pretreatment, 10, 20 and 30 min ischaemia and every 20 min through out reperfusions. All groups received treatment through 30 min ischaemia. A) Heart rate (HR); B) Mean arterial pressure (MAP); C) Rate-pressure product (RPP). Large symbols represent means ± SE for each group. Surviving rats: saline-control, n=5; AL solution, n=7; Ado-only, n=4; Lido-only, n=6.

Figure 6. Scatterplot of the relationship of MAP and RPP and infarct size following pretreatment prior to ischaemia. Negative values connote the decline in the measured points. Following pretreatment, a correlation was found between infarct size and all hemodynamic variables in the Ado-only treatment group. The applicant found that a greater decrease in MAP ($R^2 = 0.96$, $p = 0.020$) and RPP ($R^2 = 0.98$, $p = 0.012$) correlated with a larger infarct size. There was no significant effect on infarct size by hemodynamic changes in either the saline-control groups, AL solution or the Lido-only pretreatment.

Figure 7. The episodes and duration of ventricular tachycardia (VT) and ventricular fibrillation (VF) and VT+VF during ischaemia for surviving rats of second study. These values represent overall sum of episodes and durations (sec) that occurred throughout the 30 min ischaemic period. Surviving rats: saline-control, n=5; AL solution, n=7; Lido, Ado-SEQ, n=5; AL SEQ, n=6, AL-Pre-I-Rep, n=6. *P<0.05 vs. control, †P<0.05 vs AL solution group.

Figure 8. Effects of AL solution and sequential administration of adenosine and lignocaine during ischaemia and/or reperfusion on infarct size. (A) Areas at risk (AAR) were not significantly different between groups. (B) Areas of necrosis (AN/LV) in the left ventricle were reduced with AL solution treatment in comparison all groups tested (C). Infarct sizes (AN/AAR) in groups receiving AL treatment were significantly smaller compared with all other groups. Surviving rats: saline-control, n=5; AL solution, n=7; Lido, Ado-SEQ, n=5; AL SEQ, n=6, AL-Pre-I-Rep, n=6. *P<0.05 vs. Control; †P≤0.05 vs. AL solution.

Figure 9. Preconditioning: Deaths from ventricular fibrillation during ischemia. The actual percentage of animals that died per group is shown above bars. The total number of rats in each group are as follows: Saline-control, n=12; IPC, n=6; AL soln, n=7; A1 agonist (CCPA, 5μg/kg) plus lido; n=6 A1 agonist only (CCPA, 5μg/kg), n=7.

Figure 10. Preconditioning: The episodes and duration of ventricular tachycardia (VT) and ventricular fibrillation (VF) and VT+VF during ischemia for surviving rats in all treatment groups. These values represent the overall sum of episodes and durations (sec) that occurred throughout the 30 min ischemic period. Surviving rats: saline-control, n=5; IPC, n=5, AL soln, n=7, A1 agonist (CCPA, 5μg/kg) plus lido, n=6 A1 agonist only (CCPA, 5μg/kg), n=5. *P<0.05 vs. control; †P<0.05 vs. IPC.

Figure 11. Preconditioning: Effects of IPC, AL soln, A1 agonist (CCPA) plus lidocaine, and A1 agonist (CCPA) only on left ventricle necrosis and infarct size. Areas at risk (AAR/LV) were not significantly different between groups (A). Areas of necrosis in the left ventricle (AN/LV) were significantly smaller with AL mixture treatment (A). Infarct sizes (AN/AAR) in groups receiving AL solution treatment were significantly smaller compared with all other treatment groups (B). Surviving rats: Saline-control, n=5; IPC, n=5; AL soln, n=7; A1 agonist (CCPA, 5μg/kg) plus lido, n=6; A1 agonist only (CCPA, 5μg/kg), n=5. *P<0.05 vs. control.

Figure 12. Schematic of adenosine and lidocaine's possible mutiple signaling mechanisms involved in early (classic) preconditioning of the *in situ* rat myocardium and coronary microvascular. Co-administering adenosine (or adenosine agonists) plus lidocaine target electrophysiological (nodal, intercalated discs, myocyte), mechanical and metabolic sites which lead to substantial protection against mortality, life-threatening arrhythmias and tissue necrosis. Delayed protection is due in part to improved atrial and ventricular matching of electrical conduction and pump performance. Targeting adenosine receptors and voltage sensitive Na^+ fast channels may offer a new therapeutic window to delay myocardial damage during ischemia-reperfusion. **Abbreviations used:** AP, action potential; AV, atrioventricular; Gi/o, inhibitory membrane bound G protein which

couples adenosine receptors to intracellular signaling pathways; PKC, protein kinase C; $IK_{Ach/Ado}$, inwardly rectifying K^+-channel current which in supraventricular tissue (e.g., AV nodal myocytes) is directly linked to activation by adenosine/A1 activation (cAMP independent) - hyperkalemia potentiate slowing AV nodal conduction [40]; SR, sarcoplasmic reticulum; ROS, reactive oxygen species which in small amounts may serve as signal transduction messengers; cyclic AMP, cyclic adenosine monophosphate; AL, adenosine and lidocaine.

EXAMPLES

Example 1: Combinational therapy of adenosine and lignocaine after regional ischemia (at varying concentrations).

Animals and Reagents: Male Sprague Dawley rats (330-400g) from the James Cook University Breeding Colony were fed *ad libitum* and housed in a 12-hour light/dark cycle. On the day of the experiment rats were anaesthetised with an intraperitoneal injection of Nembutal (Sodium Pentabarbitone; 60 mg/kg) and the anaesthetic was administered as required throughout the protocol. Animals were treated in accordance with the James Cook University Guidelines for use of 'Animals for Experimental Purposes' (Ethics approval number A557). Adenosine (A9251 >99% purity), copper II pthalocyanine-tetrasulfonic acid tetrasodium salt (blue dye), and triphenyltetrazolium chloride (TTC) and all chemicals were obtained from Sigma Aldrich (Castle Hill, NSW). Lidocaine hydrochloride was purchased as a 2% solution (Ilium) from the local Pharmaceutical Supplies (Lyppard, Queensland).

Surgical Protocol: Anesthetized animals were positioned in a specially designed plexiglass cradle. A tracheotomy was performed and the animals were artificially ventilated at 75-80 strokes per min on humidified room air using a Harvard Small Animal Ventilator (Harvard Apparatus, Mass., USA). Blood pO_2, pCO_2 and pH were maintained in the normal physiological range and measured on a Ciba-Corning 865 blood gas analyser. Body temperature was maintained at 37°C using a homeothermic blanket control unit (Harvard Apparatus, Mass., USA). The left or right femoral vein was cannulated using PE-50 tubing for drug infusions

while the left femoral artery was cannulated for blood collection and to monitor blood pressure (UFI 1050 BP) using a MacLab.

A left thoracotomy was performed through the 4th and 5th intercostals space. The pericardium was opened and the heart gently exteriorized. A 6-0 suture was threaded under the left coronary artery (LCA) located between the base of the pulmonary artery and left atrium. The LCA ties were attached to a custom designed snare occluder fastened to the cradle via a 20-inch teflon tube attached to a detachable 10 g weight. By adding or removing the weight, a constant ligation pressure could be applied and easily released. Leads were implanted subcutaneously in a lead II electrocardiogram (ECG) configuration. Rats were stabilised for 15-20 minutes prior to occlusion. Any animal that produced dysrhythmias or a sustained fall in mean arterial blood pressure below 80 mmHg was discarded from the study. Ischaemia was confirmed by regional cyanosis downstream of the occlusion and reperfusion was confirmed by lack of cyanosis in that region.

Experimental Design: The protocols are summarised below and in Fig. 1. The experimental work was conducted over a period of approximately three hours. This comprised 5 minutes "pre-treatment", immediately following the 20 minute equilibration period referred to above. At the end of the pre-treatment period, the left coronary artery was ligated. This was maintained for 30 minutes to cause ischaemic conditions, followed by 30 minutes of reperfusion and another 2 hours of reperfusion, after which the risk area and infarct size measurements were taken. In Study I, the timing of administration of the compositions was a key parameter being tested. As shown in Figure 1, and stated in the experimental methods, constant infusion was carried out during the 5 minutes of pre-treatment and the 30 minutes of ischaemia. This was the protocol used for infusion of saline controls, AL solution, Ado only (305 micrograms/kg/min iv) and Lido only (608 micrograms/kg/min iv). The other three administration protocols were as follows. "Sequential bolus/infusion" ("Lido, Ado SCQ") involved a bolus dose of Lido at the end of the pre-treatment period (2 mg/kg iv) followed by another similar bolus at the end of the 30 minute ischaemic period. One minute before this second bolus, Ado infusion was commenced continuously through to the end of the 30 minute

reperfusion period at 150 microgram/kg/min iv. "Sequential AL Infusion" (or "AL SEQ") involved two infusions of AL solution, the first being for a 5 minute pre-treatment period, and the second being for about 35 minutes comprising the last 5 minutes of the 30 minutes of ischaemia and the 30 minutes of reperfusion (again using the 305 Ado and 608 Lido microgram/kg/min iv doses). Finally, "Constant AL Infusion" (or "AL Pre-I-Rep") involved continuous AL infusion for a period of about 65 minutes from the beginning of the pre-treatment period to the end of the 30 minute reperfusion period at the same doses (Ado 305 and Lido 608 microgram/kg/min iv).

Study I: The adenosine and lidocaine solution (AL solution) contained 6.3 mg/ml adenosine (Ado) and 12.6 mg/ml lidocaine (Lido) and was prepared on the day of the experiment in physiological saline (0.9%). Drugs were infused intravenously at 1 ml/hr (210 infusion pump, Stoelting, Illinois), which convert to mass specific dosages of 305 µg/kg/ml/min and 608 µg/kg/ml/min for Ado and Lido respectively. In the first study, 36 animals were randomly assigned into 4 treatment groups: (1) Saline-controls (0.9% saline) (n=12); (2) AL solution (n=8), (3) Ado-only (305 µg/kg/ml/min, n=8); or (4) Lido-only (608 µg/kg/ml/min, n=8). All rats received continuous infusion for 5 minutes prior to and throughout 30 minutes of regional ischaemia. The treatment was ceased when the coronary ligature was released at the onset of reperfusion after 30 min ischaemia and animals reperfused for 120 minutes for infarct sizing.

Study II: Rats (n = 19) were randomly assigned to one of three different treatment regimes: (1) Lido, Ado SEQ: a rapid bolus of lidocaine (2 mg/kg i.v.) given 1 min before LCA ligation and another bolus at 1 min before reperfusion. In addition, adenosine (150 µg/kg/ml/min) was infused 2 min before reperfusion and continued throughout 30 min of reperfusion (n=7). (2) AL SEQ: AL given at two separate times, 5 min before but not throughout ischaemia then 5 min before reperfusion and throughout 30 min reperfusion (n=6); (3) AL Pre-I-Rep: AL 5 min before and throughout ischaemia and 30 min reperfusion (n=6). These groups were compared to saline-controls and the AL solution group from Study I.

The primary end-points used to assess the cardioprotective effects of AL solution were infarct size, episodes and duration of ventricular arrhythmias and death. High mortality in the control group was observed in these pilot studies as is common in the rat model of acute myocardial ischaemia [84]. On the basis of the binomial distribution for episode of ventricular fibrillation cited in the Lambeth Conventions, the study protocol required at least 4 animals in each group to survive for sufficient statistical power to test the primary end-points [85]. The secondary end-points included heart rate, mean arterial pressure (systolic pressure – diastolic pressure/3 + diastolic pressure) and rate pressure product (heart rate x systolic pressure).

Analysis of the ischaemic area at risk and infarct size: After 120 minutes reperfusion, the coronary artery was reoccluded and the heart excised. Blue dye (Copper (II) Pthalocyanine-tetrasulfonic acid Tetrasodium salt, 3 ml) was flushed retrograde through the aorta at a flow rate of approximately 18 ml/min and allowed to circulate through the coronary vasculature to delineate the ischaemic risk zone. The heart was sliced transversely into 6 or 7 slices of uniform thickness (2mm) using a custom-made, equal spaced, multi-scalpel blade slicer. The slices were weighed and digitally photographed. Area measurements were made using the Image J (NIH) image analysis program. The area left unstained by the blue dye was defined as the left ventricular 'area-at-risk' (AAR/LV) while the blue-stained region was the perfused area not at risk of suffering ischaemic damage. The slices were then incubated in a 1% solution of triphenyltetrazolium chloride (TTC) at 37°C for 15 min [86], immersed in formalin and photographed again. The area of necrosis in the left ventricle (AN/LV) was the region of the slice unstained by TTC (white) while the non-infarcted region was the area of the slice stained by TTC (brick red). Infarct size of the left ventricle was defined as the ratio of the area of necrosis (AN) to the area at risk (AN/AAR) and expressed as a percentage.

Arrhythmia Analysis: Arrhythmias were analysed separately during 30 min ischaemia and the first 30 min of reperfusion. Using the lead II ECG tracing, the episodes and duration of episodes of ventricular tachycardia (VT) and ventricular fibrillation (VF) were recorded. Ventricular tachycardia was defined as 4

or more consecutive ventricular premature beats [85]. VF was defined as a signal where individual QRS deflections could not easily be distinguished from each other and where rate could no longer be measured [85]. Episodes referred to the number of episodes of VT or VF. The duration of each episode was recorded in seconds and the sums of these were analysed. To overcome the occasional difficulty of identifying VT or VF, the frequency and duration of both were summed and analysed separately. For example, a VT with *torsade de pointes* morphology that converted to VF then reverted to VT without a clear-cut interface was included in the summed measurement [84]. Notwithstanding this limitation, every attempt was made to identify VT and VF as separate variables.

Statistical Analysis: All values were expressed as means ± SE of the mean. For infarct size data, a one-way analysis of variance (ANOVA) was used with a least significance difference (LSD) post hoc test. A Mann-Whitney *U* test was used for comparison of arrhythmia frequency and duration because the variables of VT and VF are not normally distributed [84]. Hemodynamic data (heart rate, mean arterial pressure and rate-pressure product) was compared using an ANOVA for repeated measures. Statistical significance was defined as a P value of ≤ 0.05.

Results

Three rats were excluded from the study: one animal's MAP was <70 mmHg before treatment (Lido-only), a second animal's ventilation tubing became clogged (AL solution group), and a third rat from Lido-only group died before the end of the experiment from severe hypotension; no ventricular arrhythmias were involved. Data from a total of 52 rats is reported and the mean body weight was 361 ± 3 g. No significant differences in rat weights were found between the groups.

For Study I described above, mortality data are summarised in Fig 2. Seven of the 12 (58%) saline-control rats and 4 of the 8 (50%) Ado-only treated rats died during the ischaemic period from an episode of ventricular fibrillation. No

deaths occurred in the Lido-only treated rats (n=6) or in AL solution infused animals (n=7) (Fig 2). Only data from surviving rats were further analysed.

The mean number of episodes of ischaemia-induced VT in saline-controls was 18 ± 9 affecting 100% of animals (Fig. 3a), and 40% experienced VF (4 ± 3 episodes). Treatment with Ado-only resulted in VT in 50% of the rats tested (11 ± 7 episodes) and 100% of rats had VF (3 ± 2 episodes). In Lido-only treatment, ventricular tachycardia occurred in 83% (23 ± 11 episodes) and VF in 33% (2 ± 1 episodes) of rats tested. In AL solution treated rats, 57% of subjects had at least 1 episode of VT (2 ± 1) while no rats experienced a single episode of VF (Fig. 3a).

There were no significant differences in duration of arrhythmias in the Ado-only or Lido-only treatments compared to saline-controls, or to each other (Fig 3b). Rats infused with AL solution experienced not only a significant reduction in VT's, but also a significant reduction in durations of VT (2 ± 1 sec) and VT+VF's (2 ± 1 sec) compared to saline-controls. The durations of VT and VT + VF's for saline-controls were 106 ± 45 sec and 156 ± 72 sec and for Lido-only treatment were 31 ± 18 sec and 37 ± 22 sec respectively (Fig 3b). In addition, infusion of AL solution significantly reduced the durations of the VT episodes compared to Ado-only treated rats (27 ± 18 sec) (Fig. 3b). It was noted that, with the exception of the AL solution group, a high variability in arrhythmia frequency and duration across treatment groups was observed (Fig. 3). Only the infusion of AL solution provided consistent reductions of VT or VF frequency or duration without large variability between samples.

In respect of reperfusion arrhythmias, within the first minute of reperfusion, 80% of saline-controls, 75% of the Ado-only and 16% of Lido-only treated animals experienced at least one episode of VT of 0.6 to 35 sec duration. Neither treatment with Ado-only or Lido-only differed significantly from each other, or from saline-controls ($P<0.05$). An episode of VF occurred in 1 of the 5 saline-controls within the first minute and lasted 16 sec. There were no episodes of VF in Ado-only or Lido-only treatment groups during 30 min reperfusion. Rats treated with AL solution experienced no ventricular arrhythmias (VT or VF) at or during reperfusion. The number of episodes of VT from saline-controls and the Ado-only

treated animals was found to be significantly higher than AL solution treated animals. Additionally, the durations of VT and VT+VF durations in the Ado-only group (11 ± 8 sec for both) were significantly longer than treatment with AL solution.

Mean area at risk as a proportion of the left ventricle (AAR/LV), areas of necrosis (AN/LV) and infarct size (AN/AAR) expressed as a percentage of left ventricle are shown in Fig 4 (a) to (c). The areas at risk for saline-controls, Ado-only, Lido-only and AL solution treated animals were 63 ± 7%, 58 ± 8%, 56 ± 8% and 48 ± 8% respectively, and not significantly different ($P < 0.05$). Overall, the mean risk area was 55 ± 4% (n=22) (Fig 4a). The areas of necrosis for saline-controls, Ado-only and Lido-only animals were 38 ± 5%, 33 ± 7% and 33 ± 3% respectively, and were not significantly different from each other (Fig 4b). In contrast, the area of necrosis in AL solution treated animals was 18 ± 4% and significantly lower all other treatments (Fig 4b). Similarly, the mean infarct size was reduced in rats infused with AL solution (38 ± 6%) compared to saline-controls (61 ± 5%) ($P<0.05$), Lido-only treated animals (66 ± 8%) ($P<0.05$), and the Ado-only group (56 ± 5%) ($P=0.06$) (Fig 4c). There was no significant difference between mean infarct sizes between saline-controls, Ado-only, or Lido-only treatments.

Heart rate (HR), mean arterial pressure (MAP) and rate-pressure product (RPP) are shown in Fig. 5 (a) to (c) , respectively. There were no significant differences among groups prior to pretreatment. Pretreatment of either AL solution or Ado-only resulted in equivalent decline in MAP and RPP while MAP and RPP of saline-controls and Lido-only treated animals were similarly elevated. Both Lido-only and AL solution resulted in bradycardia while Ado-only and saline treatment maintained heart rate throughout ischaemia. The dramatic drop in heart rate shown at 10 min ischaemia in the saline-control group was associated with ventricular fibrillation during that time (Fig. 5a). Otherwise, heart rate was sustained in saline-controls. Although AL solution treatment resulted in decreased hemodynamics throughout ischaemia, the decline of both MAP and RPP was not statistically different from other treatments. Only at the end of 30 min ischaemia

did the RPP between treatments diverge. Saline-controls and Ado-only treatment rose to levels statistically higher than AL solution and Lido-only treatment.

At reperfusion, coinciding with the discontinuation of treatment, hemodynamics in all groups rose toward pretreatment values. However, within the 120 min reperfusion period, no treatment reached starting baseline values in any group. Despite this, AL solution treatment resulted a significant improvement in MAP by the end of 120 min reperfusion compared to all treatment groups.

Evaluation of hemodynamics from all groups indicated no correlation between infarct size and MAP or RPP at pretreatment or assessed every 5 min during 30 min ischaemia. However, to ensure that individual treatments' hemodynamic changes did not lead to reduced infarct sizes instead of the treatment itself, a correlation analysis was performed on individual group MAP and RPP changes following pretreatment (Fig 6a and 6b). Hemodynamic changes from saline-control and the Lido-only pretreatment did not significantly affect infarct size. Treatment with Ado-only resulted in a correlation between the reduction in hemodynamics from pretreatment and infarct size. The more Ado-only treatment reduced MAP or RPP, then the greater the infarct size (MAP, $R^2 = 0.96$, $p = 0.020$; RPP, $R^2 = 0.98$, $p = 0.012$). Pretreatment with AL solution led to infarct size reduction which was independent of changes in MAP or RPP ($p \geq 0.60$), despite the dramatic decrease in all hemodynamic variables accompanying pretreatment with AL solution.

While Lido-only and AL solution treatment led to similar RPP and MAP responses throughout ischaemia (Fig 5), the effect of these treatments on infarct size were opposing. Treatment with AL solution decreased infarct size by nearly 42% from controls while Lido-only treatment resulted in an infarct size increase of about 8% above saline-controls (Fig 4), yet both groups showed no significant differences in hemodynamic properties during ischaemia.

The results of Study II directed to the effect of sequential administration of AL solution or adenosine and lidocaine during ischaemia and/or reperfusion follow. Mortality data is summarised in Fig. 2. Pretreatment with a 2 mg/kg lidocaine

bolus resulted in two deaths from ventricular fibrillation during ischaemia before adenosine infusion commenced (Lido, Ado SEQ, n=7). In contrast, no deaths occurred from ischaemia-induced arrhythmias in rats pretreated with 5 min of AL infusion, which was resumed for 5 min before reperfusion and continued during 30 min reperfusion (AL SEQ) (see Figs. 1 and 2). Similarly no deaths were recorded in animals continuously infused with AL for 5 min pretreatment, 30 min ischaemia and 30 min reperfusion (AL-Pre-I-Rep) (Fig. 1).

The episodes and durations of VT and VF from rats that survived ischaemia are shown in Fig. 7. Forty per cent of the lidocaine-pretreatment group (Lido, Ado SEQ) experienced 6 ± 3 episodes of VT of 4 ± 2 sec duration and 1 ± 0 episodes of VF of 1 ± 0 sec duration during ischaemia (before adenosine infusion) (Fig 7). The sum of VT and VF episodes and durations for these groups were 7 ± 3 and 4 ± 2 sec respectively. The lidocaine pretreatment strategy (Lido, Ado SEQ) did not significantly reduce episodes or durations of VT or VF compared to saline-controls. In contrast, animals infused with AL during 5 min pretreatment and continued throughout 30 min ischaemia and reperfusion experienced significantly reduced episodes and durations of VT (2 ± 1, 2 ± 1 sec, 57% affected) and VT+VF (2 ± 1, 2 ± 1 sec) compared to saline-controls (18 ± 9, 106 ± 45 sec, 100% affected and 22 ± 12, 156 ± 72 sec, respectively) ($P<0.05$). However, a 5 min pretreatment of AL solution discontinued during ischaemia (AL SEQ) was not sufficient to prevent VF episodes (2±1, 21±8 sec, 67% affected), or reduce VT (39 ± 23, 84 ± 49 sec, 83% affected) and VT+VF (40 ± 23, 104 ± 46 sec). Importantly, only constant infusion of AL solution before and during ischaemia prevented episodes of VF during ischaemia.

Animals pretreated with a 2 mg/kg bolus of lidocaine followed by another lidocaine bolus and adenosine infusion (Lido, Ado SEQ) before reperfusion experienced 6 ± 3 arrhythmia episodes of 7 ± 4 sec duration at reperfusion (48% VT and 52% VF). Neither the number of VT+VF episodes nor the durations of these were significantly different from saline-controls ($P<0.05$). In contrast, AL given as pretreatment and for 30 min ischaemia resulted in significantly fewer early reperfusion-induced arrhythmias than the separate and sequential infusions of lidocaine and adenosine (Lido, Ado SEQ). Likewise, there were no reperfusion-

Induced arrhythmias in animals given AL solution in any sequence (AL SEQ, AL-Pre-I-Rep) resulted in significantly reduced episodes in comparison to saline-controls ($P<0.05$).

Mean area at risk, areas of necrosis and infarct size (as for Study I) are shown in Fig 8. The areas at risk for lidocaine-bolus/adenosine infusion (Lido, Ado SEQ), sequential AL infusion (AL SEQ) and constant AL infusion (AL-Pre-I-Rep) were 55 ± 5% , 44 ± 8% and 47 ± 6% respectively. No significant differences were found in risk areas among the different treatment groups and controls (63 ± 7%) (Fig 8). The area of necrosis for Lido, Ado SEQ (29 ± 4%) and AL SEQ (29 ± 5%) were not significantly different from saline-controls. In contrast, pretreatment with AL solution continued through ischaemia and reperfusion (AL-Pre-I-Rep) significantly reduced left ventricular necrosis (21 ± 6%) compared to controls (38 ± 5%) (Fig. 8). The mean infarct size for Lido, Ado SEQ, AL SEQ and AL-Pre-I-Rep treated groups were 52 ± 5%, 67 ± 8%, and 41 ± 10% respectively and not significantly different from saline-controls (61 ± 5%) ($P<0.05$). In contrast, when AL was infused continuously for 5 min before and during 30 min ischaemia (results in first study), significant reductions in infarct size (38 ± 6%) were found when compared to either AL SEQ or saline-controls ($P<0.05$) (Fig. 8).

Heart rate, MAP and RPP at the end of equilibration, after 5 min pretreatment, at 25 minutes ischaemia, 30 minutes reperfusion, 60 minutes reperfusion and 119 minutes reperfusion for the second experiment were analysed. There were no significant differences among groups prior to any treatment. At pretreatment little change occurred to the hemodynamics in the saline-controls and lidocaine-bolus treatment group (Lido, Ado SEQ). Animals receiving the two different AL protocols (AL SEQ, AL-Pre-I-Rep) experienced a significant reduction in all hemodynamic variables at pretreatment, and also at 25 min ischaemia compared to saline-controls or Lido, Ado SEQ group ($P<0.05$). By 119 min reperfusion MAP was significantly improved in the Lido, Ado-I/R SEQ (86 ± 10 mmHg) compared with groups where AL solution was given for 30 min reperfusion (AL SEQ 66 ± 5, and AL Pre-I-Rep, 69 ± 3) ($P<0.05$).

Collectively, these results show the effects of adenosine and lidocaine continually infused either individually or combined in solution during ischaemia in an *in vivo* rat model of regional myocardial ischaemia. In particular, an intravenous infusion of adenosine and lidocaine solution before and during ischaemia offers superior protection from death, arrhythmias and tissue necrosis than either drug alone or when lidocaine bolus preceded adenosine infusion. Further, the sequential administration of lidocaine followed by adenosine during ischaemia and/or reperfusion was inferior to administration of the AL solution as pretreatment and throughout ischaemia, as measured by protection from mortality, arrhythmias and ultimately infarct size.

The infusion of AL solution resulted in no deaths in the four protocols and 26 animals tested (Fig. 1 and 2). In contrast, 58% of the saline-controls, 50% of the Ado-only treated animals, and 29% of the animals receiving a 2 mg/kg bolus of lidocaine died during ischaemia from ventricular fibrillation (Fig 1 and 2). Given adenosine's well-known role to potentiate the abolition of catecholamine triggered ventricular arrhythmias [87,88], and the nucleoside's ability to reduce myocardial injury when administered prior to and during regional or global ischaemia [22,89-92], it was surprising that the adenosine only infusion failed to protect from death. Adenosine may have either failed to protect the heart from arrhythmias or, based on the higher relative durations of VF compared to durations in saline-controls, may have promoted arrhythmias. The applicant believes that death during ischaemia with adenosine infusion has not been reported before in the rat, dog, pig or human. Thus, these results were unexpected. It seems unlikely that they relate to the concentration administered. Lee et al. infused similar concentrations of adenosine (250-350 µg/kg) for 10 min in humans prior to elective cardiopulmonary bypass surgery without untoward effects [93]. Indeed, it was found that adenosine pretreatment improved post-bypass left ventricular function compared to no treatment, and that benefit continued 40 hours postoperatively [93]. Arrhythmias were not investigated. Higher doses of adenosine have been used in other surgical settings without adverse effects. Lagerkranser et al., used a dose range of 60 - 350 µg /kg/min i.v. in patients undergoing surgery for cerebral

aneurism and found that adenosine-induced hypotension (MAP of 40-50 mmHg) did not affect cerebral oxygenation unfavourably [94].

In contrast to saline-controls and the Ado-only treatment, rats infused with Lido-only experienced no arrhythmias that resulted in death (Fig 2). However, when a rapid bolus of lidocaine was given prior to ischaemia, 29% of the animals died despite comparatively low episodes and durations of arrhythmias among surviving animals (Fig 6). These deaths occurred in the ischaemic period before the second bolus of lidocaine and adenosine infusion commenced (Lido, Ado SEQ group; see Fig 1). While the early work of Homeister et al., [29] did not study the effect of lidocaine or adenosine on mortality rates, they did exclude 6 dogs that had received a rapid bolus of lidocaine (2 mg/kg I.v.) because of intractable VF, and five saline-controls [29]. Presumably, these subject exclusions died during ischaemia. In the study, infusion of a lidocaine bolus failed to reduce arrhythmias. The combination of adenosine and lidocaine in AL solution, however, was outstanding among all other treatments in consistently abolishing ventricular fibrillation. Even when AL solution was only applied at pretreatment there were no episodes of death, despite a variable amount of arrhythmias during ischaemia (Fig. 6).

Likewise during reperfusion, rats receiving Ado-only, Lido-only or lidocaine bolus/adenosine infusion group (Lido, Ado SEQ) experienced VT or VF early during reperfusion. Again, rats infused with AL solution experienced no early reperfusion-induced arrhythmias in any of the four protocols and 26 animals tested (see results). Without being bound, the applicant speculates that the genesis of early reperfusion-induced arrhythmias may be related to oxygen-derived free radicals [95], and that AL solution attenuated the formation of reactive oxygen species such as hydrogen peroxide or free radical generation. While both adenosine and lidocaine alone have been shown to be protective against reactive oxygen species [96,97], the separate and sequential infusion of lidocaine and adenosine failed to stop such arrhythmias in the study.

Rats treated with AL solution before and during ischaemia had infarct sizes significantly lower (38 ± 6%) than saline-controls (61 ± 5%), Ado-only (56 ± 5%)

and Lido-only treatment (66 ± 8%) (Fig 4). If AL solution was continued through 30 min reperfusion the infarct size reduction was virtually unchanged (41 ± 10%) compared to AL infusion during pretreatment and 30 min ischaemia (38 ± 6%) (Fig 4). When AL solution was administered only at pretreatment and then again 5 min before reperfusion, no deaths occurred, but the mean infarct size was larger (67 ± 8%) than saline-controls. These results support the conclusion that, for infarct size reduction, AL solution must be applied at least at pretreatment and throughout the ischaemic period. Moreover, when lidocaine was given as a pretreatment bolus followed by another bolus and adenosine infusion prior to reperfusion (Lido, Ado SEQ), the infarct size was high (52 ± 5%), again reinforcing the conclusion that sequential treatments of adenosine and lidocaine are not as effective as a constant infusion of a mixture of adenosine and lidocaine in solution.

On the basis of the relationship between aortic diastolic pressure, coronary perfusion pressure, and myocardial oxygen supply, it was expected that any treatment-induced decrease in hemodynamic variables from pretreatment and throughout ischaemia would correlate with infarct size reduction. However, the study failed to show a statistical difference between a decrease in MAP or RPP and reduced infarct size. Indeed, treatment with adenosine only resulted in the reverse: higher infarct sizes were associated with lower MAP and RPP. Its arguable that the limited number of samples that survived for infarct sizing in the Ado-only group may have reduced the power of that data; however, a large percentage of the animals given Ado-only died from VF during ischaemia during treatment. Overall, this indicates that adenosine alone was incapable of protecting the myocardium, regardless of the limited number of infarct sizes that could be assessed.

On the whole, a reduction in infarct size was observed only in the AL solution group (Fig 4). Particularly interesting, the Lido-only and AL solution treatment groups incurred similar MAP and RPP during ischaemia, yet infarct size outcomes were the most widely separated. The infarct sizes resulting from ischaemia were more greatly reduced in the AL solution than from Lido-only treatment, which appeared to not protect from infarction. Similarly, sequential administration of AL (AL SEQ), infused once at pretreatment and then again just

before reperfusion, resulted in reduced hemodynamics similar to AL solution. However, the data clearly showed that protection from arrhythmias and infarct expansion was not achieved without continuing AL treatment during ischaemia. Therefore, lowering demand or work on the heart with AL solution did not appear to play a role in reducing infarct size or arrhythmias in this study.

Without being bound by any theory or mode of action, it is believed that protection is related to the synergistic effect of adenosine and lidocaine combined to reduce calcium entry into the myocardial cell. A mechanistic synergy between adenosine and lidocaine action may occur that affords the myocardium protection. This data imply that each drug amplifies the effect of the other leading to a reduction in infarct size, episodes of ventricular arrhythmias and death compared to the administration of either drug alone. For example, it is known that Ca^{2+} overload in the ischaemic myocardium predisposes the tissue to injury in part by disturbing membrane linked ionic homeostasis and maintenance of the membrane potential which can lead to high incidences of arrhythmias [98,99]. Reducing intracellular Ca^{2+} overload is likely due to a complex interaction between adenosine and lidocaine targets involving the opening the A1-mediated ATP-sensitive potassium channels (K_{ATP} channels) [22] whilst blocking sodium (Na^+) channels having the overall effect of reducing Na^+ entry and the activity of Na^+/Ca^{2+} exchanger [100,101]. In addition, these actions may enhance cAMP-linked attenuation of VT [102]. Furthermore, that no reperfusion arrhythmias were found in any of the AL solution treated rats, demonstrates that protection extended into the reperfusion period. Yoshida et al [103] have shown in humans that reperfusion VT are most likely arrhythmias triggered by cAMP mediation rather than re-entrant electrical circuits. Whereas Lu et al. [100] have attributed inhibition of Ca^{2+} loading by lidocaine's blocking Na^+ entry which appears more prominent in ischaemic tissue thereby synchronizing myocardial cells and making reentrant arrhythmias less likely. Therefore, the AL solution in the study may have provided a primary window to reduce triggered (adenosine) and re-entrant (lidocaine) arrhythmias through an amplified reduction of cytosolic Ca^{2+} during ischaemia-reperfusion.

AL cardioprotection may also relate to the collective action of both drugs in reducing the inflammation response to injury. Adenosine is a potent modulator of the anti-inflammatory response by strongly inhibiting the activation of neutrophils, platelets and mononuclear leukocytes, which can lead to cytoxicity and endothelial dysfunction [22,104-106]. Additionally, Zhao et al. [107] have linked adenosine infusion at reperfusion with reduced PMN accumulation and reduced myocardial apoptosis. Recent work by Nakamura et al. [108] corroborated this finding in rat hearts by showing that PMN accumulation was significantly correlated with the number of apoptotic cells. Lidocaine also modulates a Na-channel independent inflammatory response by inhibiting the priming of human neutrophils and superoxide anion production with a suspected target site in a G_q–coupled signalling pathway [109,110]. Additionally, lidocaine inhibits intracellularly coupled lysophosphatidic acid (LPA) signalling [69]. LPA is an intercellular phospholipid mediator with multiple actions linked to stimulation of inflammatory events such as platelet aggregation and neutrophil activation. As these events are related to the development of anatomic no reflow, AL solution may play a part initially reducing functional damage from ischaemic injury and hinder the progression of anatomic no reflow [111-113]. The effects of AL combination to reduce ischaemia-reperfusion injury may also be linked to reducing the adverse effects of the inflammatory process which includes attenuating the production of free radicals, reducing capillary plugging and minimising direct injury to cardiomyocytes.

Accordingly, AL solution administered 5 min before and during 30 min regional ischaemia resulted in no deaths, lower episode of ventricular arrhythmias and lower infarct size in the *in vivo* rat model of regional ischaemia. The cardioprotective properties of AL solution during ischaemia and reperfusion may involve opening the A1 receptor-linked K_{ATP} channels, blocking the Na^{2+} fast channels, adenosine and lidocaine's combined effect on cAMP mediated attenuation of ventricular arrhythmias, and suppression of the inflammatory response to injury. Focusing primarily on a pharmacological therapy for reperfusion injury may deny the underlying cause of the injury and its effective treatment. While minimizing reperfusion injury with adenosine has been a focus in recent years, treatment with AL solution before and during ischaemia reinforces

the concept that ischaemia and reperfusion are composite events requiring an integrated strategy to optimize protection of an organ or tissue.

It is known that rat studies have differences from clinical scenarios because of differences in mass-specific metabolic rate [114], differences in electrophysiological properties [115] and functional morphology such as collateral circulation [116 117]. There is substantial data as to how such translation studies apply to the clinic. Because of the higher metabolic rate in the rat and the extremely short half-life of adenosine (8 sec) [52], the applicant chose upper range adenosine concentrations that have led to improved function or reduced necrosis in animal models [35,118] as well as provided a therapeutic benefit to humans [93,94]. The main problem limiting adenosine's use in humans is its hypotensive effect but this concern can be minimized during surgical procedures or in the clinical setting when adenosine can be administered as an intracoronary bolus or infusion [119]. In humans, intracoronary infusions of up to 240 µg/min adenosine causes minimal decrease in arterial pressure, heart rate or electrocardiographic variables [119]. Similarly, intracarotid injections of adenosine of 1000 µg/ml in baboons has a profound effect to increase cerebral blood flow without any significant systemic side effects [120]. In relation to lidocaine, the maximum safe dose of lidocaine for humans is approximately 4 mg/kg i.v. (without epinephrine) and 7 mg/kg i.v. (with epinephrine). Lidocaine also has a short plasma half-life of approximately 8 minutes. Overall, a 70 kg adult should not receive more than around 300-500 mg cumulative dose of lidocaine. For convenience, the example of the invention given above omitted the standard rapid bolus of lidocaine (1-2 mg/kg) that usually precedes a continuous infusion [29-31,121] and opted for a lower dose (608 µg/kg/ml/min) continuous infusion. Additionally, using lidocaine this way was intended to avoid the reported pro-arrhythmic effects of lidocaine [122]. Another precaution in comparing data on rats and humans are differences in collateral circulation of the heart. However, since humans have a greater collateral circulation than the rat [117], superior cardioprotection by AL infusion is expected to have a greater effect in human patients.

Example 2: The effect of the Pharmacological Preconditioning the Heart: Targeting Adenosine receptors and voltage-sensitive Na^+ fast channels.

Example 1 investigates the preconditioning effect of combinatorial therapy targeting adenosine receptors and voltage-dependent sodium fast channels in the *in situ* rat model of regional ischaemia. Adenosine and/or A1 receptor agonist (CCPA) plus lidocaine was co-administered 5 min before and during 30 minutes coronary artery ligation, and the results compared to classical ischaemic preconditioning. Adenosine and lidocaine is used in example 1 as the sole arresting and protecting combination in cardioplegia, and that co-administration of the two drugs at non-arresting concentrations during ischaemia result in better cardioprotection. Cardiac Na^+ channels initiate and propagate action potentials in the atria, ventricles and intercalated discs, and their gating is believed to rely exclusively on changes in the resting membrane potential [123,124]. During ischemia, reduced excitability leads to a rise in extracellular K^+, a less negative membrane potential and a decreased inward Na^+ current (I_{Na}) which in turn shortens the epicardial action potential duration, reduced Ca^{2+} entry and helps to protect the heart from arrhythmias.

Methods: Rats (n=38) were randomly assigned to one of five groups: (1) Saline controls (0.9% saline) (n=12); (2) IPC (n=6); (3) AL soln (n=7); (4) A1 agonist plus lidocaine (n=6). (5) A1 agonist (CCPA, 5µg/kg) (n=7). Ischemic preconditioning was achieved using 3 cycles of ischemia/reperfusion with each transition lasting 3 min (Group 2). The adenosine and lidocaine solution (AL soln) was prepared on the day at mass specific dosages of 305 µg/kg/min and 608 µg/kg/min respectively (Group 3). Group 1 and Group 3 rats received continuous infusion of saline or AL soln, respectively, for 5 min before and throughout 30 min of regional ischemia. At the onset of reperfusion the treatment was ceased. Group 4 rats were pretreated 5 min before ligation with a 5 min bolus of A1 agonist CCPA (5µg/kg) alongside a continuous infusion of lidocaine (608 µg/kg/ml/min) which was continued throughout 30 min ischemia. Group 5 was treated with A1 agonist (CCPA) alone 5 min before ligation. All animals were reperfused for 120 min for infarct sizing. The primary end-points were death, episodes and duration of ventricular arrhythmias and infarct size. Hemodynamics constituted the secondary end-points (heart rate,

mean arterial pressure and systolic pressure). Infarct size is considered the "gold standard" of ischaemic preconditioning.

Results: Mortality data are summarized in Fig 9. Seven of the twelve saline-controls, one of the seven ischemic preconditioned (IPC) rats and two in the CCPA-treated group (n=8) died during 30 min ischemia from ventricular arrhythmias. In contrast, none of the adenosine and lidocaine-treated rats (n=7) or CCPA plus lidocaine-treated rats (n=6) died (Fig. 10). Only data from surviving rats were further analyzed.

Episodes and duration of ventricular tachycardia or fibrillation during 30 min ischemia are shown in Fig. 10. Saline controls had 156±72 sec of ventricular arrhythmias (VT, 106±45; VF, 49±30), and CCPA-treated animals had 56±18 sec of VT with virtually no fibrillation (Fig. 10). Forty percent of the IPC treated-rats experienced 4±3 episodes of VT for over 8±6 sec. Preconditioning with AL abolished VF and significantly reduced episodes and durations of VT with the average duration of the VT 2±1 sec from controls (106±45 sec). Within the AL-treated group, 42% of animals did not experience VT or VF (Fig 10). Treatment with CCPA plus lidocaine completely abolished VT and VF in all animals tested (Fig. 10). Immediately following ischemia, 80% of saline-controls, 60% of IPC-treated, and 100% of CCPA-treated rats experienced reperfusion tachycardias (Data not shown). No ventricular arrhythmias during reperfusion were experienced in rats preconditioned with AL or CCPA plus lidocaine (Fig 10).

The mean area at risk per left ventricle (AAR/LV), areas of necrosis (AN/LV) and infarct size (AN/AAR) are shown in Fig 11. The areas at risk expressed as a percent of the left ventricle were not significantly different among the five groups, and on average comprised 58 ± 2% (Fig. 11). The areas of necrosis in saline-controls, AL soln, A1 agonist (CCPA) alone, IPC and A1 plus lido-treated rats were 38 ± 5%, 16 ± 4%, 24 ± 3%, 7 ± 2% and 8 ± 3%, respectively. These measurements translated into a mean infarct size 61 ± 5% for saline–controls, 38 ± 6% for AL soln treated rats, 42 ± 7% for A1 agonist (CCPA) treated animals, 11 ± 3% for IPC treated animals and 12 ± 4 for CCPA and lidocaine-treated rats (Fig

11). IPC and pharmacological preconditioning with CCPA and lidocaine-treated rats were not significantly different (P<0.05) (Fig 11).

Table 1. Heart rate and mean arterial blood pressure

Treatment		Baseline	Ischemia Start	30 min Ischemia	120 min Reperfusion
Saline-controls	HR (bpm)	436 ± 13	433±15	391±31	381±30
	MAP (mmHg)	112±6	110±11	77±11	62±8
	Systolic (mmHg)	139±6	137±11	104±6	86±12
IPC	HR (bpm)	438±9	416±16	414±7	379±8
	MAP (mmHg)	130±8	90±19	92±14	69±6
	Systolic (mmHg)	163±12	116±22	116±14	98±6
AL soln	HR (bpm)	497±13	332±14*†	316±17*†	395±11
	MAP (mmHg)	123±11	46±4*†	52±6†	66±6
	Systolic (mmHg)	159±11	75±7*†	86±8†	119±7
A1 agonist (CCPA) plus lidocaine	HR (bpm)	436±18	270±14*†	172±26*†	347±17
	MAP (mmHg)	110±12	49±4*†	44±2*†	72±5
	Systolic (mmHg)	131±9	77±7*†	59±3*†	97±9
A1 agonist (CCPA) only	HR (bpm)	421±15	336±29*†	308±73	367±12
	MAP (mmHg)	114±6	89±10	91±10	71±2
	Systolic (mmHg)	146±6	113±11	113±12	94±4

Data are mean ± S.E.M.; *P<0.05 vs. control. †P<0.05 vs. IPC

The hemodynamic changes during pretreatment, ischemia and reperfusion are found in Table 1. Rats treated with AL or CCPA plus lidocaine had significant reductions in heart rate, MAP and systolic pressure compared to saline-controls and IPC. No signficant differences in MAP were apparent between AL or CCPA plus lidocaine, although heart rate was lower in the latter (Table 1). Though all groups' hemodynamic measurements were lower than baseline after 2hrs reperfusion, no group was significantly different from another.

Discussion: Ischemic preconditioning (IPC) remains one of the most potent means of cardioprotection known. Nearly every IPC study has shown a profound reduction in infarct size, and most have reported a large reduction in the incidence of arrhythmias; while others, including the original study of Murry et al., 41,125, have shown that IPC may have a proarrhymic effect and increase the possibility of stunning (Metzner, Yellon). The results in this example demonstrate that pretreating the *in situ* rat heart with adenosine and lidocaine (AL), or with adenosine A1 agonist (CCPA) and lidocaine, 5 min before and 30 min during acute regional ischaemia, results in no deaths, no lethal arrhythmias and a large decrease in infarct size compared to saline-controls. The most surprising result was that infarct size reduction in CCPA plus lidocaine-treated rats (12±4 %) matched that of ischemic preconditioning (11± 3%) demonstrating that the combination of adenosine A1 subtype activation and down-regulation of voltage-dependent Na^+ fast channels was as effective at reducing infarct size as IPC. Moreover, the combination of A_1L (and AL) surpassed IPC protection in having no deaths and abolishing ventricular arrhythmias (Figs 9 and 10).

Without being bound by any theory or mode of action, it is believed that Adenosine or adenosine A1 agonists with lidocaine protect the myocardium and coronary microvascular at three levels; electrophysiological, mechanical and metabolic. The results in this example demonstrate that ventricular arrhythmias were significantly reduced. Again, without being bound by any theory or mode of action, this is believed to be due to the combination of the composition according to the invention having improved atrial and ventricular matching of electrical conduction and pump performance. Adenosine activates A1 receptors and thus are considered to be involved in slowing the sinoatrial nodal pacemaker rate

(negative chronotropy), delaying atrioventricular (A-V) nodal impulse conduction (negative dromotropy), reducing atrial contractility (negative inotropy), and inhibits the effect of catecholamines (via reduction in cyclic AMP and inhibition of Ca^{2+} influx) [75,126]. Adenosine is 30 times more effective in slowing the conductance of A-V nodal than SA pacemakers [127], which may be more important to terminate abnormal arrhythmias in combination with lidocaine's ability to reduce the voltage dependent Na^+ entry and resetting membrane potential to a more polarised voltage (i.e. limit the reduction in ischemic-induced maximum diastolic potential). Lidocaine's pharmacological effects on electrical conduction and excitability are particularly pronounced during ischemia [62]. Lidocaine binds to the intracellular side of the Na channel near the inactivating gating domains. Our results demonstrate that improved atrial and ventricular matching may be associated with the combined actions of adenosine and lidocaine to downregulate the heart by shortening action potential duration and reduce contractility which would allow less time available for Ca^{2+} entry via L-type channels, and by increasing the diastolic duration interval which may involve a reduced maximum negative membrane potential reached during diastole, a longer slope of phase 4 depolarisation, and a change to the threshold at which an action potential fires. Membrane hyperpolarisation or the slowing of depolarisation in the presence of AL would effectively reduce Na^+ and Ca^{2+} entry during ischaemia and protect the cells from arrthymias. Since adenosine receptors and sodium channels are also located in intercalated discs [83], reduced membrane excitability may also reduce gap-junction coupling which would further benefit atrial-ventricular matching of conduction and pump performance. A1 activation leads to delayed protection by delaying the rise of intracellular Na^+ and Ca^{2+}. This has been demonstrated in rat myocytes and human cell line (tsA201) [128]. Reduced Na^+ and Ca^{2+} entry would also decrease axial resistance and improve electrical conduction in ischemic hearts [128]. Furthermore, the probable reduction of atrial and ventricular myocyte excitability, delayed repolarization and therefore increased refractoriness by adenosine receptor stimulation with lidocaine may be linked with a decrease in re-entrant ventricular arrhythmias, particularly in the highly vulnerable epicardial ischemic zone.

Pretreating the heart with adenosine or A1 agonist with lidocaine resulted in significant cardioprotection as judged by the "gold standard" of infarct size reduction (Fig 11). Adenosine is thought to be involved in myocardial preconditioning [36,37]. Adenosine A1 receptor activation (and in some cases A3) has been implicated in the rat [39], rabbit [36,129], dog [130], pig [103] and human [75,131]. The present study supports the role of CCPA A1 activation to reduce infarct size in the rat model (Fig 11). Adenosine's role as a 'trigger' of preconditioning has been supported from studies using the non-selective receptor antagonist 8-(p-sulphophenyl)-theophylline (SPT) which reduces protection a number of animals models [37,129]. Adenosine A1-receptors, like bradykinin and opiod receptors, are known to confer protection via inhibitory G protein-coupled pathways which have been linked to the opening of sarcolemma ATP sensitive K^+ channels [132] Adenosine A1 receptor 'trigger' activation has also been linked to new targets including the mitochondria [126,133-135] and sarcoplasmic reticulum. [136] Nevertheless, it remains to be established how the opening the mitochondrial K_{ATP} channel and/or reactive oxygen species 'triggers' and/or mediates the delay of cell injury and how the different K_{ATP} channels relate to one another, and other potential 'triggers' to reduce infarct size by preconditioning the heart. Without been bound by any theory or mode of action, figure 12 summarise our model of adenosine and lidocaine's possible multiple signalling mechanisms involved in early (classic) preconditioning of the in situ rat myocardium and coronary microvascular.

Lidocaine also has a history of reducing acute regional ischemia in heart and brain [123,138-140]. Low concentrations of lidocaine bind to amino acids positioned on the intracellular side of the Na^+ channel near the inactivating gating domains [141], and are potentiated by ischaemia [142]. The shift in the Na^+ channel's voltage-dependence to a more polarised state compared to ischaemia alone, and lidocaine's ability to inhibit L-type calcium channels (ref), help explain the drug's anti-ischemic actions to delay Na^+ and Ca^{2+} entry into the cell [Haigney, 1994 #1372 [82]. Lidocaine's anti-ischemic effects might also be enhanced by adenosine's anti-adrenergic actions to indirectly inhibit the Na^+/H^+ [143] and Na^+/Ca^{2+} exchangers [144]. Thus, due to the central role of voltage-gated Na^+ channels in modulating Ca^{2+} entry, lidocaine with adenosine or A1 agonist would be expected to delay Na^+ entry and reduce Ca^{2+} loading. Lidocaine and

adenosine also have potent anti-inflammatory properties which without being bound to any theory or mode of action may explain the low number of arrhythmias during ischemia, and particularly in the reperfusion period (Fig 10). Both adenosine and lidocaine are known to attenuate neutrophil activation [22,97] and inhibit platelet activation and plugging. [22,69]

We have demonstrated that infarct size in AL treated rats falls from 61% to 38%. Since the mean arterial pressure (MAP) was not significantly different between AL and A_1L treatments (Table 1), the contribution of hypotension to infarct-size reduction in our rat model cannot exceed the fall from 61 to 38% (Fig 3). Thus the infarct size reduction from 38% to 12% in the A_1L treated rats must be due to factors other than hypotension. In this case, the maximal contribution of hypotension to infarct reduction would be 47% [(61-38)/(61-12) x 100] in CCPA + L treated rats, with the remaining 53% coming from the pharmacological therapy itself. If hypotension contributed to 50% of the infarct reduction in the AL-treated animals compared to controls, then the direct benefit of the drug combination CCPA + L would be nearly 77%. We conclude there that the direct cardioprotection from A1 + L-treated rats is at least 53%. However, it has been shown that hypotension on its own does not reduce infarct size. In 1997 Casati et al., showed in the *in vivo* rabbit model that the protective action of A1 receptor activation by CCPA was independent of changes to hemodynamics including MAP [145]. In this study atenolol (a Dadrenoceptor blocker), felodipine (a Ca^{2+} channel blocker) and A2A selective agonist (2-hexynyl-5'-N-ethyl-carboxamindoadenosine, 2HE-NECA) and 5'-N-ethyl-carboxamindoadenosine (non-selective adenosine agonist, NECA) reduced MAP similar to CCPA but did not change infarct size [145]. In addition, in separate studies the bradycardia effect of CCPA (Table 1) has been shown to contribute little to infarct size reduction. By pacing isolated rat hearts, De Jong and colleagues showed that CCPA was still cardioprotective in paced hearts compared to hearts without pacing. We demonstate that infarct size reduction in CCPA + L-treated rats is largely due to the combined mechanism of action, not to hemodynamic effects [37].

Accordingly, a composition according to the invention has been shown to provide a composition to use as an alternative method to 'classical' ischemic

preconditioning involving physical clamping of the heart. The results in this example show that co-administration (i.v.) of the A1 receptor agonist CCPA and Na^+ fast channel modulator lidocaine 5 min before and during 30 min of left coronary artery ligation results in no deaths, no arrhythmias and a profound reduction in myocardial infarct size which was not significantly different to ischaemic preconditioning. Targeting adenosine A1 receptor subtype and Na^+ fast channel modulation may offer a new therapeutic window to delay myocardial damage during ischemia and improve left contractile function in reperfusion (Fig 12). In the clinical setting, adenosine-lidocaine preconditioning therapy may be useful in arrhythmia management and could be administered via an intracoronary route for open-heart surgical procedures or for angioplasty where acute systemic hypotension is to be avoided [42,48]. More importantly this demonstrates that the preconditioned effect of A1 adenosine receptor agonist is not limited to Adenosine and Lignocaine but can include the other potassium channel openers and/or adenosine receptor agonists, (including indirect adenosine receptor agonists).

Example 3: Effect of Adenosine and Lignocaine with Esmolol on functional recovery of the rat heart after arrest

This example demonstrates the effect of esmolol, an antiadrenergic, together with Adenosine and Lignocaine on functional recovery after a period of arrest using intermittent perfusion.

Hearts from adult whistler rats (350g) were prepared using the method described below.

Intermittent retrograde perfusion was performed under a constant pressure head of 70mmHg after hearts were switched back from the working mode to the Lagendorff mode. After stabilisation, the hearts were arrested using either:

(i) Adenosine (200uM) and Lignocaine (500uM) plus Esmolol (100uM);

(ii) Adenosine (200uM) and Lignocaine (500uM) plus Esmolol (10uM);

(iii) Adenosine (20uM) and Lignocaine (500uM) plus Esmolol (100uM).

Solutions containing these compounds were provided in Krebs Henseleit (10nM glucose, pH 7.55 @ 37°C). The aorta was then cross-claimed and the heart left to sit arrested for 5 mins, after which the clamp was released and 2 mins of arrest solution delivered from a pressure head of 70mmtg. The clamp was replaced and this procedure continued for 18mins arrest time then 30mins arrest time. The recovery results are shown in Tables 2 to 4 below.

This example demonstrates improved functional recovery of the heart after 30mins arrest, providing superior protection during arrest and recovery of the heart.

Throughout this specification, unless stated otherwise, where a document, act or item of knowledge is referred to or discussed, this reference or discussion is not an admission that the document, act or item of knowledge, or any combination thereof, at the priority date, was part of the common general knowledge.

Table 2: Functional parameters of isolated working rat hearts during pre-arrest and reperfusion following 30min arrest with adenosine-lignocaine plus esmolol. Adenosine (200 uM), Lignocaine (500 uM)and Esmolol (100 uM) (in 10 mM glucose containing Krebs Henseleit, pH 7.55 delivered intermittently at 37°C)

30 min Arrest Protocol	n	Time to Arrest (sec)	Cardioplegia flow (ml/min)	Heart Rate (bpm)	Systolic/ diastolic Pressure (mmHg)	Aortic Flow (ml/min)	Coronary Flow (ml/min)
PRE-ARREST (5 min)	1			295	120/70	33	15.5
ARREST 5 min Induction		14 sec	10	ARREST	ARREST	ARREST	ARREST
@18 min (for 2 min)			3	ARREST	ARREST	ARREST	ARREST
@30 min (2 min)			4.5	ARREST	ARREST	ARREST	ARREST
RECOVERY							
15 min				225	110/70	10.5	13.5
30 min				246	110/75	12	12
45 min				223	110/78	12.5	11

Table 3: Functional parameters of isolated working rat hearts during pre-arrest and reperfusion following 30min arrest with adenosine-lignocaine plus esmolol. Adenosine (200 uM), Lignocaine (500 uM)and Esmolol (10 uM) (in 10 mM glucose containing Krebs Henseleit, pH 7.60 delivered intermittently at 37°C)

30 min Arrest Protocol	n	Time to Arrest (sec)	Cardioplegia flow (ml/min)	Heart Rate (bpm)	Systolic/ diastolic Pressure (mmHg)	Aortic Flow (ml/min)	Coronary Flow (ml/min)
PRE-ARREST (5 min)	1			244	115/65	35.5	16
ARREST 5 min Induction		23 sec	9	ARREST	ARREST	ARREST	ARREST
@18 min (for 2 min)			7	ARREST	ARREST	ARREST	ARREST
@30 min (2 min)			6	ARREST	ARREST	ARREST	ARREST
RECOVERY							
15 min				184	120/60	33	16.5
30 min				255	90/70	5	12
45 min				271	85/65	3	10

Table 4: Functional parameters of isolated working rat hearts during pre-arrest and reperfusion following 30min arrest with adenosine-lignocaine plus esmolol. Adenosine (20 uM), Lignocaine (500 uM)and Esmolol (100 uM) (in 10 mM glucose containing Krebs Henseleit, pH 7.51 delivered intermittently at 37°C)

30 min Arrest Protocol	n	Time to Arrest (sec)	Cardioplegia flow (ml/min)	Heart Rate (bpm)	Systolic/ diastolic Pressure (mmHg)	Aortic Flow (ml/min)	Coronary Flow (ml/min)
PRE-ARREST (5 min)	1			206	110/70	21	10
ARREST 5 min Induction		25 sec	5	ARREST	ARREST	ARREST	ARREST
@18 min (for 2 min)			4	ARREST	ARREST	ARREST	ARREST
@30 min (2 min)			2.5	ARREST	ARREST	ARREST	ARREST
RECOVERY							
5 min				119	120/60	9	4
15 min				154	100/70	4	5
45 min				154	90/70	4	2

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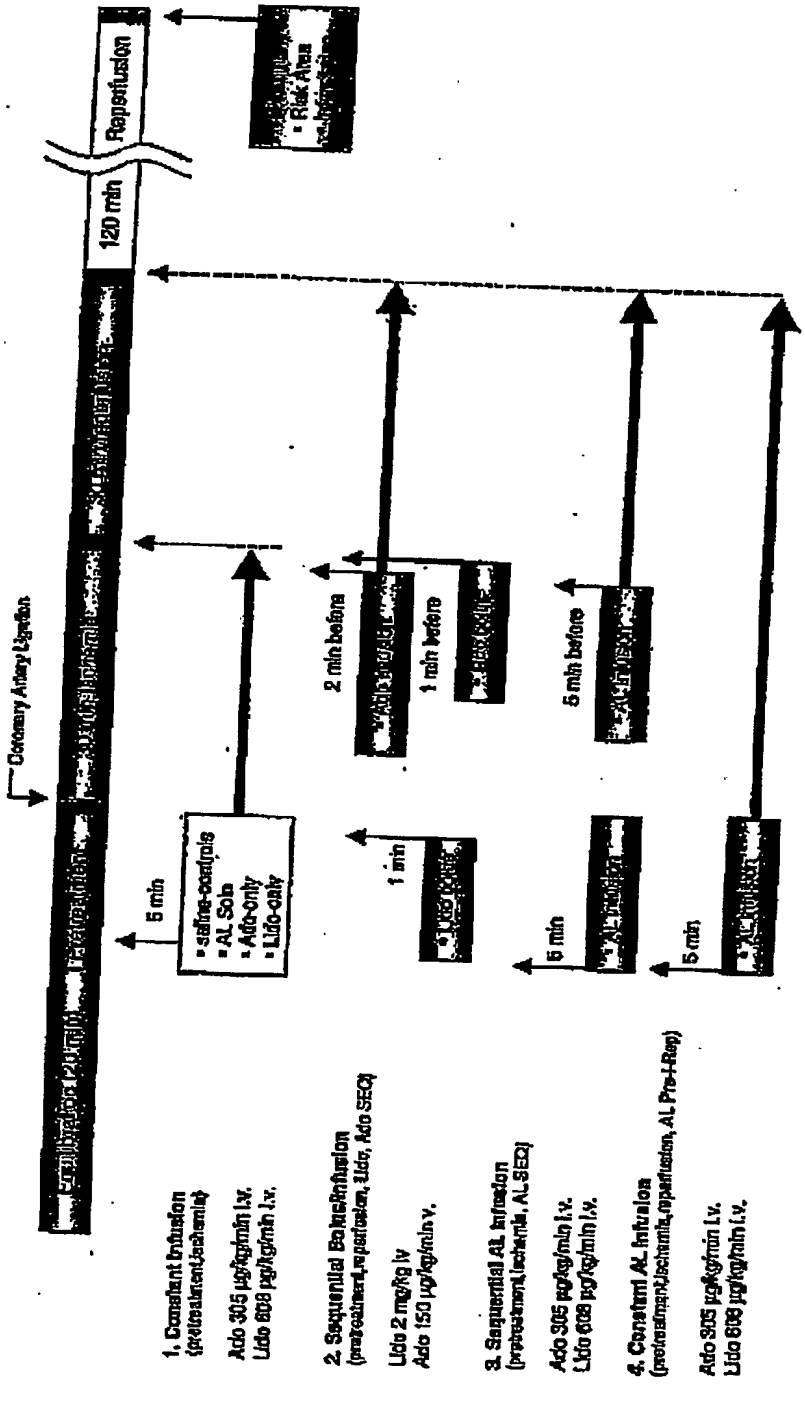

Reperfusion
120 min
Risk Area
Coronary Artery Ligation
5 min
• saline-controls
• AL Solo
• Ado-only
• Lido-only
1. Constant Infusion
(pretreatment,ischemia)
Ado 305 µg/kg/min i.v.
Lido 608 µg/kg/min i.v.
2 min before
1 min before
1 min
2. Sequential Bolus/Infusion
(pretreatment,reperfusion, Lido, Ado SEQ)
Lido 2 mg/kg iv
Ado 150 µg/kg/min v.
5 min before
5 min
3. Sequential AL Infusion
(pretreatment,ischemia, AL SEQ)
Ado 305 µg/kg/min i.v.
Lido 608 µg/kg/min i.v.
5 min
4. Constant AL Infusion
(pretreatment,ischemia,reperfusion, AL Pre-I-Rep)
Ado 305 µg/kg/min i.v.
Lido 608 µg/kg/min i.v.

Figure 1

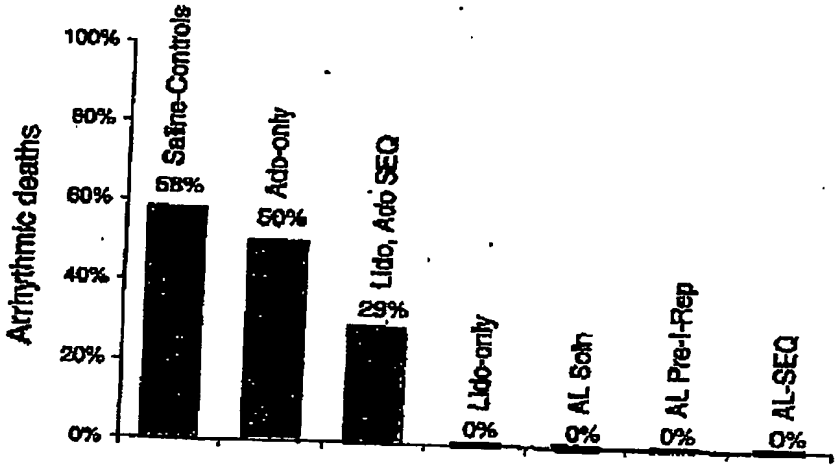

Arrhythmic deaths
100%
80%
60%
40%
20%
0%
Saline-Controls
68%
Ado-only
50%
Lido, Ado SEQ
28%
Lido-only
0%
AL Soln
0%
AL Pre-I-Rep
0%
AL-SEQ
0%

Figure 2

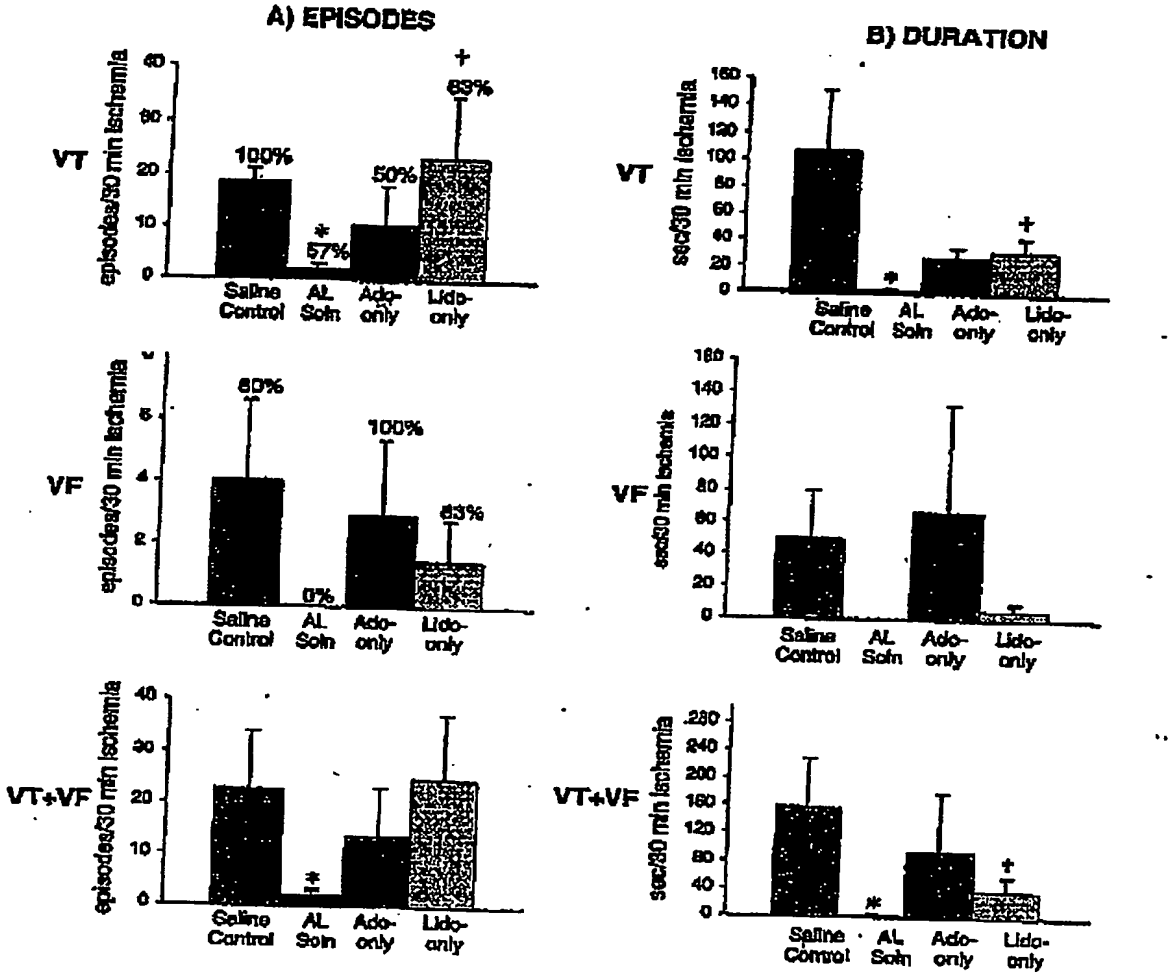

A) EPISODES
B) DURATION
VT
VF
VT+VF
episodes/30 min ischemia
sec/30 min ischemia
100%
57%
50%
83%
80%
0%
100%
83%
Saline Control
AL Soln
Ado-only
Lido-only

Figure 3

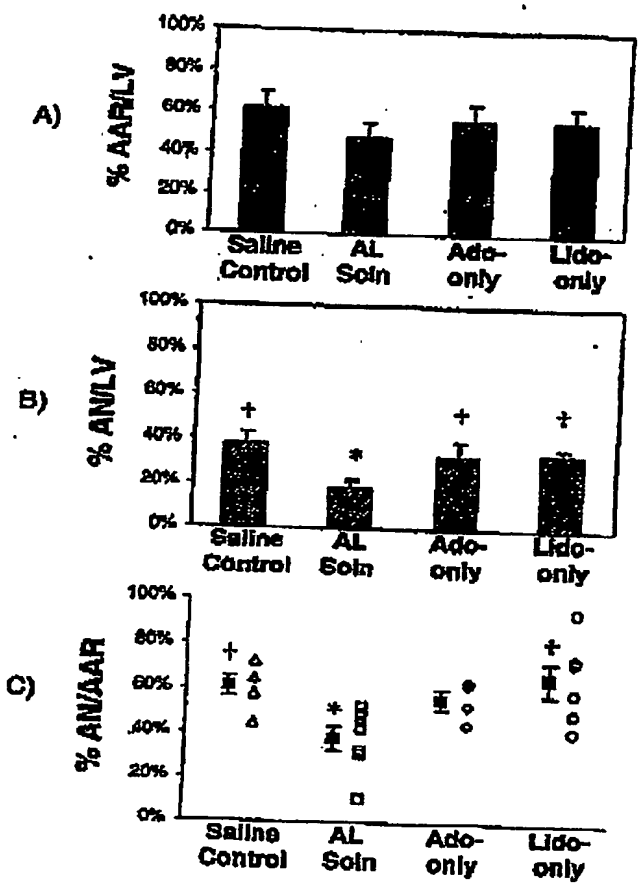

A)
% AAR/LV
100%
80%
60%
40%
20%
0%
Saline Control
AL Soln
Ado-only
Lido-only
B)
% AN/LV
100%
80%
60%
40%
20%
0%
Saline Control
AL Soln
Ado-only
Lido-only
C)
% AN/AAR
100%
80%
60%
40%
20%
0%
Saline Control
AL Soln
Ado-only
Lido-only

Figure 4

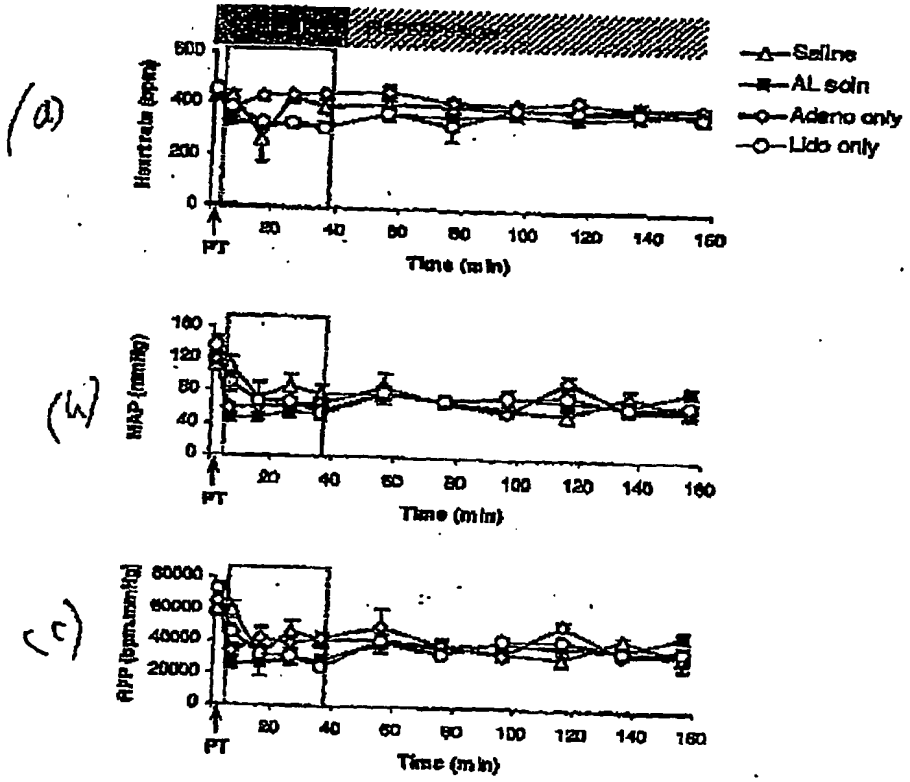

(a)
Heartrate (bpm)
600
400
200
0
PT
20
40
60
80
100
120
140
160
Time (min)
Saline
AL soln
Adeno only
Lido only
(b)
MAP (mmHg)
160
120
80
40
0
PT
20
40
60
80
100
120
140
160
Time (min)
(c)
RPP (bpm.mmHg)
60000
40000
20000
0
PT
20
40
60
80
100
120
140
160
Time (min)

Figure 5

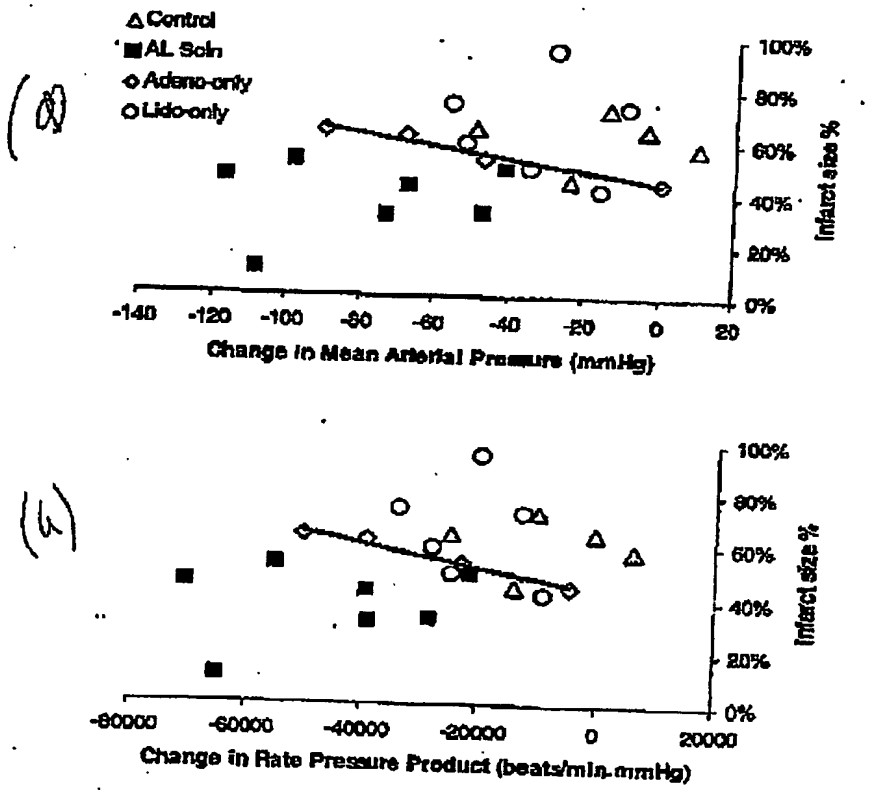

△ Control
■ AL Scln
◇ Adeno-only
○ Lido-only
100%
80%
60%
40%
20%
0%
Infarct size %
-140
-120
-100
-80
-60
-40
-20
0
20
Change in Mean Arterial Pressure (mmHg)
100%
80%
60%
40%
20%
0%
Infarct size %
-80000
-60000
-40000
-20000
0
20000
Change in Rate Pressure Product (beats/min.mmHg)

Figure 6

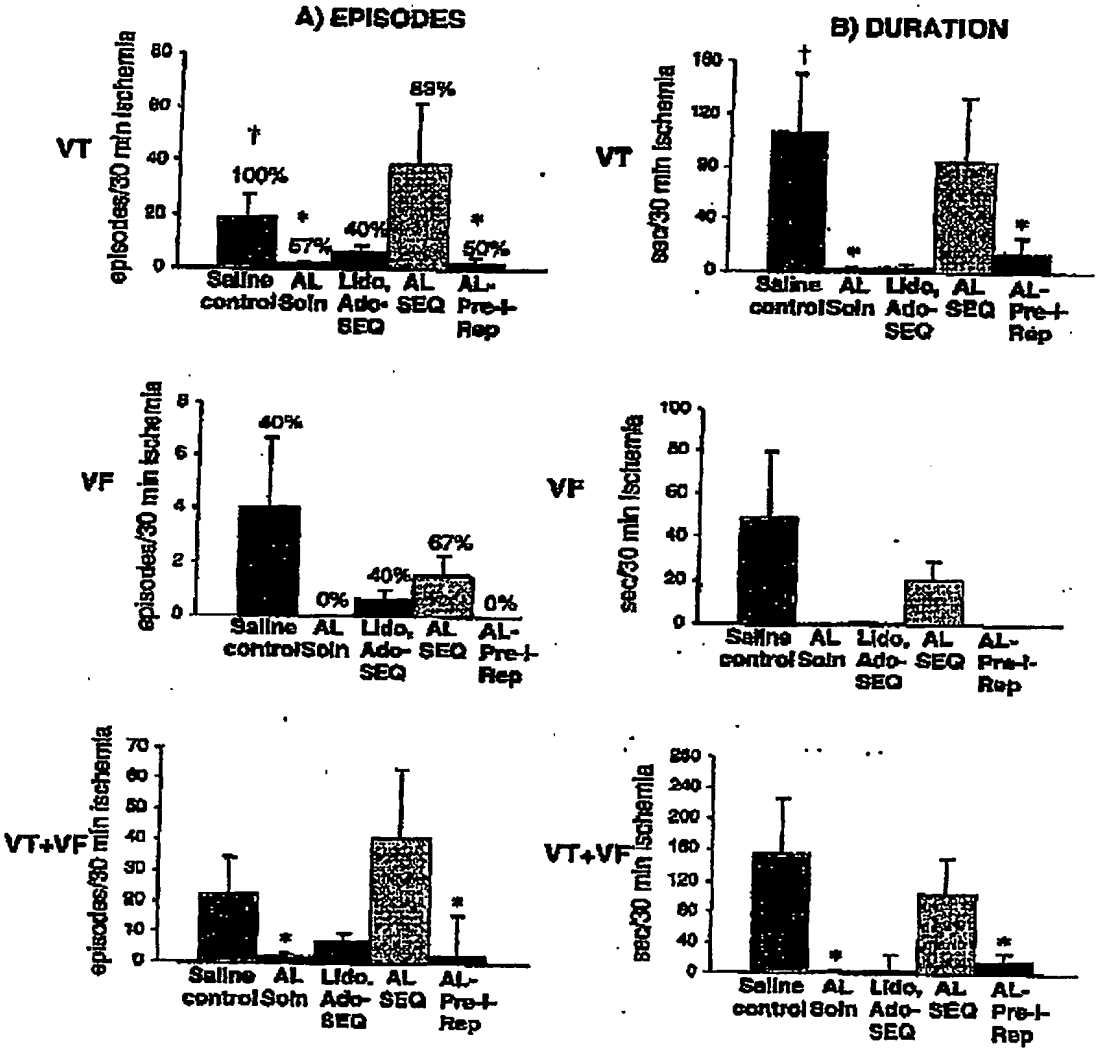

A) EPISODES
B) DURATION
VT
VF
VT+VF
episodes/30 min ischemia
sec/30 min ischemia
100%
57%
40%
89%
50%
40%
0%
40%
67%
0%
Saline control
AL Soln
Lido, Ado-SEQ
AL SEQ
AL-Pre-I-Rep

Figure 7

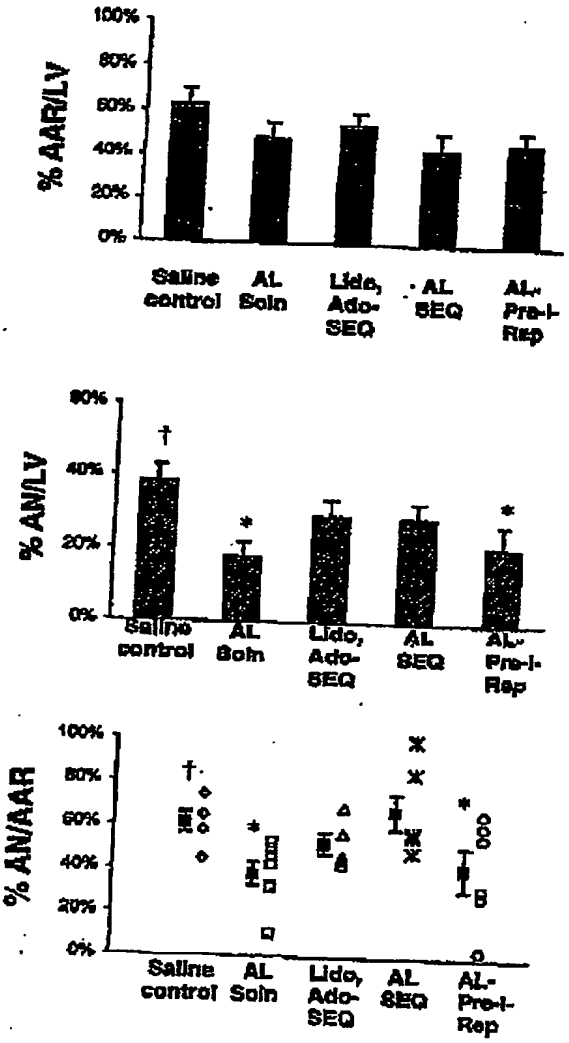

% AAR/LV
100%
80%
60%
40%
20%
0%
Saline control
AL Soln
Lido, Ado-SEQ
AL SEQ
AL-Pre-I-Rep
% AN/LV
†
*
% AN/AAR

Figure 8

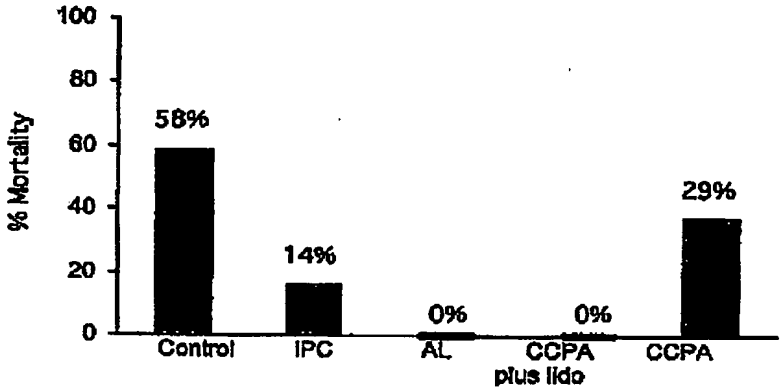
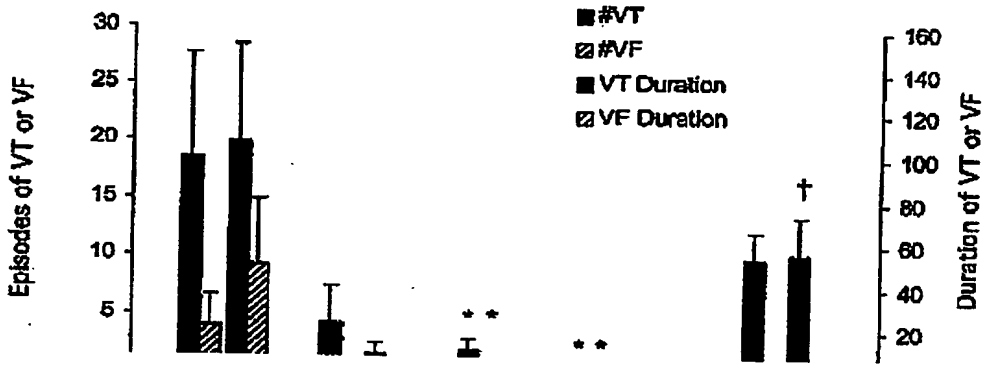

100
80
60
40
20
0
% Mortality
58%
14%
0%
0%
29%
Control
IPC
AL
CCPA plus lido
CCPA

Figure 9


Episodes of VT or VF
30
25
20
15
10
5
#VT
#VF
VT Duration
VF Duration
160
140
120
100
80
60
40
20
Duration of VT or VF
†
* *
* *